論 文 の 内 容 の 要 旨

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論文題目

Plasmid-host functional interaction network: global proteome dynamics and molecular analysis of H-NS family proteins
(プラスミド・宿主間相互作用 - プロテオーム動態および H-NS ファミリータンパク質の分子解析)

Plasmids, one of the mobile genetic elements (MGEs), play an important role in adaptation and evolution. They can confer various novel traits to their host cells, such as resistance to antibiotics and heavy metal as well as the ability to degrade xenobiotic compounds. Novel trends in plasmid research aim at understanding the molecular mechanisms underlying the functional interaction between plasmids and host cells in order to predict the behaviour of plasmid-harbouring strains in the natural environments. Using the carbazole-degradative plasmid pCAR1 as a model, our laboratory has initiated comprehensive research, which evaluates the interplay between a catabolic plasmid and several different strains from the genus *Pseudomonas*. Previous results showed that carriage of pCAR1 differentially reshaped the chromosomal gene expression and physiology of *P. putida* KT2440, *P. aeruginosa* PAO1 and *P. fluorescens* Pf0-1. However, these and other studies have characterized the plasmid-host systems mainly at transcriptional level and only few reports addressed the effect of MGEs on the protein pools of the host cells. Furthermore, the role of post-translational modification (PTM) in plasmid-host cross-talk has not been previously explored. Therefore, the first objective of this study was to evaluate the impact of pCAR1 on the proteome of the host cells using *P. putida* KT2440 as a model. In addition, two acyl lysine modifications (acetylation and succinylation) that respond to the metabolic status of the cell and regulate diverse protein properties were investigated.

Nucleoid-associated proteins (NAPs) in bacteria are global regulators of gene expression. H-NS family proteins are one of the most abundant NAPs. They share common structural organization including a C-terminal DNA-binding domain and an N-terminal dimerization/oligomerization domain, connected by a flexible linker. Previous comprehensive transcriptome and phenotypic analyses revealed that H-NS functional homologs encoded on the pCAR1 plasmid (Pmr) and the chromosome of *P. putida* KT2440

(TurA and TurB) play a pivotal role in the plasmid-host interactions. Results suggested that the three proteins cooperatively and independently regulate the expression of both chromosomal and plasmid genes. Dimerization and subsequent oligomerization are important features of the function of these proteins. Therefore, biochemical analyses and structures of plasmid- and chromosome-born H-NS homologs would provide deeper understanding of the mechanisms of plasmid effects on host-cell physiology. The structure of an oligomerization-deficient TurB truncated variant (TurB_nt_{61}-R8A), which lacks the flexible linker and the DNA-binding domain, was recently resolved (Fig.1) [1]: a central dimerization site was identified and a terminal dimerization site was predicted. The second objective of this study was characterization of the N-terminal dimerization site of TurB.

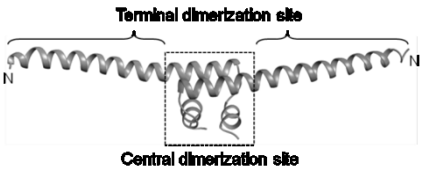

Fig. 1 Structure of TurB_nt_{61}-R8A [1].

Proteome and acylome analyses of the modification landscape created by the functional interaction between pCAR1 and host *P. putida* KT2440

In order to explore the impact of pCAR1 carriage on the protein biosynthesis of the host as well as to characterize the protein lysine acetylation and succinylation profiles of the pCAR1-free and -harbouring strains, a quantitative mass spectrometry (MS) approach based on SILAC (stable isotope labeling by amino acids in cell culture) was applied. To this end, *P. putida* KT2440 and KT2440(pCAR1) lysine auxotrophic strains (KT2440L and KT2440L(pCAR1)) were constructed and employed. To evaluate the effect of pCAR1 carriage based on the physiological state of the host, the comparative proteome profiling was performed using actively growing exponential and stationary phase cells. KT2440L and KT2440L(pCAR1) were labeled in minimal succinate medium supplemented with 'light' and 'heavy' lysine, respectively. For the total proteome analysis, equal amounts of cell-free protein extracts from both strains were mixed, digested with trypsin and subjected to MS analysis. Global acylome profiling was conducted by enrichment of acetylated and succinylated peptides from the total tryptic peptide pools using anti-acetyllysine and anti-succinyllysine antibodies.

In total, 1,592 distinct proteins were detected in the pCAR1-free and -harbouring strains: 20 proteins were encoded on the pCAR1 plasmid and 1,572 proteins were encoded on the chromosome. Carriage of pCAR1 affected 10% of the detected host proteome: 7 proteins were found to be significantly induced (more than twofold) and 151 proteins were downregulated. Notably, pCAR1 reduced the abundance of key proteins involved in central metabolism (TCA cycle and glycolysis), signal transduction and motility. The overall modest correlation between the protein abundance data and the previous transcriptomic analyses indicated the relevance of an integrated approach in order to better understand the effect of plasmid carriage on the host cells.

In total, 937 acetylation sites on 383 proteins and 331 succinylation sites on 150 proteins were reproducibly detected in two biologically-independent experiments in the pCAR1-free and -harbouring

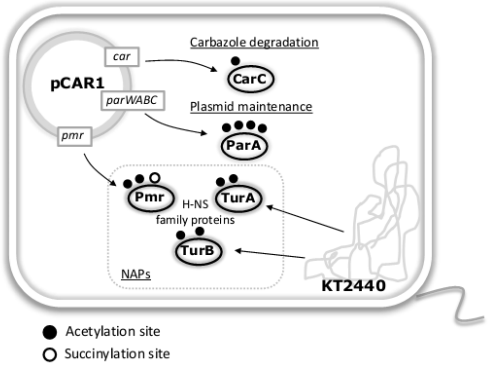

Fig. 2 Acetylation and succinylation identified on pCAR1-encoded proteins involved in carbazole degradation and plasmid maintenance and on H-NS homologs, key players in the plasmid-host cross-talk.

strains. Acetylation and succinylation mainly targeted proteins involved in translation, carbohydrate and amino acid metabolism. pCAR1 carriage resulted in changes in the acylation status of host proteins involved in the ribosome, carbohydrate and nucleotide metabolism. Furthermore, the host system invoked acetylation of important pCAR1-encoded proteins involved in carbazole degradation and plasmid maintenance (Fig. 2). Acetylation and succinylation were also detected on the pCAR1-and chromosome-encoded H-NS family proteins TurA, TurB and Pmr. Collectively, these results featured the potential of physiological cues from the host to affect plasmid-determined functions and to impact the transcriptional networks in the plasmid-harbouring systems.

Investigation of the factors affecting the acylation status in the pCAR1-free and -harbouring strains

The global proteome analyses of the pCAR1-free and -harbouring strains revealed that lysine acetylation and succinylation are abundant PTM in *P. putida* KT2440. However, the factors that affect the acylation status are still unclear. Two mechanisms for Nε-lysine acetylation have been reported in bacteria. The first relies on acetyltransferases (KATs) with acetyl-CoA as a donor of acetyl groups and the second mechanism is non-enzymatic with acetyl-phosphate (acetyl-P) serving as the acetyl group donor. Both enzymatic and non-enzymatic acetylation could be reversed by deacetylases (KDACs). Functional KATs and KDACs in *P. putida* KT2440 have not been identified yet. Bioinformatic analysis revealed more than 30 putative KATs encoded on the chromosome and one on the pCAR1 plasmid. Furthermore, *P. putida* KT2440 possesses three KDAC homologs (PP_4764, PP_5340 and PP_5402). In order to evaluate the role of the three putative KDACs, triple deletion mutants were generated (KT2440D and KT2440D(pCAR1)). To characterize also the effect of acetyl-P levels on the global acetylation status, a putative phosphotransacetylase (Pta), which catalyzes the conversion of acetyl-CoA to acetyl-P, was inactivated giving KT2440P and KT2440P(pCAR1) strains. Acetylation in the pCAR1-free and harbouring strains mainly targeted proteins involved in carbohydrate metabolism (glycolysis/gluconeogenesis, TCA cycle, pentose phosphate pathway, pyruvate metabolism), energy metabolism (oxidative phosphorylation, nitrogen, sulfur metabolism) and amino acid metabolism. Therefore, in a first set of experiments the metabolic capabilities of KT2440D and KT2440D(pCAR1) were estimated by Phenotype MicroArrays (Biolog) using 190 carbon sources, 95 nitrogen sources and 94 sulfur and phosphorous sources. KT2440D showed no pronounced difference in comparison to the control strain under all conditions tested, whereas KT2440D(pCAR1) exhibited reduction in metabolic capabilities under most conditions. The delayed growth of KT2440D(pCAR1) in comparison to KT2440(pCAR1) was further confirmed in minimal succinate medium. Deletion of the putative KDACs genes and *pta* had no dramatic effect on the global

acetylation levels of the pCAR1-free and harbouring strains, as judged by anti-acetyllysine immunoblot analyses peformed with whole-cell protein extracts from cultures grown in media supplemented with various carbon sources.

Characterization of the N-terminal dimerization site of TurB- biochemical studies and crystallization

In order to study the properties of the N-terminal dimerization site of TurB, variants truncated at the central dimerization site were constructed: TurB_ nt_{37}, TurB_nt_{40}, TurB_nt_{42} and TurB_nt_{50}. Cross-linking experiments demonstrated that all four variants formed dimers but no oligomers (results for TurB_nt_{42} and TurB_nt_{50} were published in [1]) suggesting that TurB uses both, the central and the terminal dimerization site for oligomerization. To determine the structural basis for dimerization at the N-terminal site, crystallization screening was performed with the truncated variants: TurB_ nt_{37} and TurB_nt_{40} produced no crystals, and TurB_nt_{42} produced crystals with poor diffraction. Initially, TurB_nt_{50} could not be subjected to crystallization due to formation of aggregates. Through an extensive screening 0.4 M NDSB-256 was found to prevent formation of aggregates. TurB_nt_{50} was crystallized by the hanging-drop vapour-diffusion method using a precipitant solution composed of 20% PEG3350, 0.1 M HEPES (pH 6.8), 0.1 M ammonium acetate. X-ray diffraction data were collected from TurB_nt_{50} crystals with a maximum resolution of 2.8 Å. These data are currently being analyzed.

Summary and future prospects

Through comparative proteome analyses of plasmid-free and -harbouring cells, this work determined how the catabolic plasmid pCAR1 alters the protein pools in host *P. putida* KT2440 and revealed a previously unexplored potential of metabolism-related post-translational modifications (lysine acetylation and succinylation) in the plasmid-host interactions. To examine the generality of these patterns, a broader set of host systems needs to be examined. Further studies will also be required to characterize the modulation in the pools of metabolites, whose pathways are influenced by carriage of pCAR1 in order to obtain deeper understanding of the metabolic networks in the plasmid-harbouring strains. Detailed biochemical studies are being performed to determine how acetylation and succinylation precisely affect the properties of plasmid- and chromosome-encoded NAPs. These analyses will shed light on the factors that impact the transcriptional networks in the plasmid-host systems.

In the second part of this work, the N-terminal dimerization site of the H-NS homolog TurB was characterized and crystals were obtained. Structural studies are necessary to provide more detail about the dimerization/oligomerization mechanisms of TurA and Pmr. Such information would also serve as a context for analysis of the effect of PTM on the properties of H-NS family proteins.

Reference:

[1] Suzuki-Minakuchi C, Kawazuma K, Matsuzawa J, Vasileva D, Fujimoto Z, Terada T, Okada K and Nojiri H (2016) Structural similarities and differences in H-NS family proteins revealed by the N-terminal structure of TurB in *Pseudomonas putida* KT2440. *FEBS Lett* **590**, 3583-3594.