Bioorthogonal Noncanonical Amino Acid Tagging for Selective Analysis of the *Pseudomonas aeruginosa* Proteome

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ABSTRACT

In natural environments, bacterial physiology is frequently characterized by slow metabolic rates and complex cellular heterogeneities. The opportunistic pathogen *Pseudomonas aeruginosa* provides one such example; *P. aeruginosa* forms untreatable chronic biofilm infections of the cystic fibrosis lung, where oxygen limitation can lead to states of metabolic dormancy. To better understand the biology of these states, *in vitro* experiments must be adapted to better recapitulate natural settings. However, low rates of protein turnover and cellular or phenotypic complexity make these systems difficult to study using established methods. Here we adapt the bioorthogonal noncanonical amino acid tagging (BONCAT) method for time- and cell-selective proteomic analysis to the study of *P. aeruginosa*. Analysis of proteins synthesized in an anoxic dormancy state led to the discovery of a new type of transcriptional regulator which we designated SutA. We performed detailed analyses of SutA's role in transcription under slow growth states and we elucidated the structural basis for its regulatory behavior. Additionally, we used cell-selective targeting of BONCAT labeling to determine the dynamic proteomic response of an antibiotic-tolerant biofilm subpopulation to challenge with ciprofloxacin. Overall this work shows the utility of selective proteomics as applied to bacterial physiology and describes the broad biological insight obtained from that application.

PUBLISHED CONTENT AND CONTRIBUTIONS

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NOMENCLATURE

BS^3. Bis(sulfosuccinimidyl) suberate.

Aha. L-azidohomoalanine.

Anl. L-azidonorleucine.

BONCAT. Bioorthogonal noncanonical amino acid tagging.

Bpa. L-benzoylphenylalanine.

CF. Cystic fibrosis.

ChIP. Chromatin immunoprecipitation.

DBCO. Dibenzylcyclooctyne.

GFP. Green fluorescent protein.

ICD. Isocitrate dehydrogenase.

IP. Immunoprecipitation.

LC-MS/MS. Liquid chromatography-tandem mass spectrometry.

MIC. Minimum inhibitory concentration.

ncAA. Noncanonical amino acid.

RNAP. RNA polymerase.

ROS. Reactive oxygen species.

rRNA. Ribosomal RNA.

TAMRA. Tetramethylrhodamine.

TCA cycle. Tricarboxylic acid cycle.

UTR. Untranslated region.

Chapter 1

ADAPTING AND APPLYING BONCAT TO THE STUDY OF *PSEUDOMONAS AERUGINOSA* PHYSIOLOGY

The overarching goal for my doctoral work was to adapt a method for selective proteomic analysis toward the study of bacterial physiology. When I began this effort, the bioorthogonal noncanonical amino acid tagging (BONCAT) method for proteome labeling and enrichment [1] had recently been modified to allow for cell-selective analyses [2]. BONCAT relies on the cellular incorporation of a noncanonical amino acid (ncAA) into nascent proteins. Proteins that contain the ncAA are chemically distinct from the pre-existing proteome, and the presence of a functional chemical handle on the ncAA (e.g., an azide) allows for selective chemical targeting of these proteins via bioorothogonal chemistry (e.g., azide-alkyne cycloaddition). Labeled proteins can be visualized in cells or lysates via reaction with a fluorescent tag, or can be enriched via reaction with an affinity tag or solid support followed by chromatography. Enriched proteins can then be identified and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Temporal selectivity of protein labeling is achieved with a ncAA that is incorporated by wild-type translational machinery (e.g., L-azidohomoalanine, Aha) by controlling when cells are exposed to the ncAA. Cell-selective protein labeling is achieved through the use of a ncAA (e.g., L-azidonorleucine, Anl) that is only incorporated by cells that express a mutant aminoacyl-tRNA synthetase (mRS) engineered for activity toward the ncAA. By restricting expression of the mRS to cell-types of interest, BONCAT labeling can be targeted to particular cells present in complex, heterogeneous mixtures.

Through the work of my colleagues and others, BONCAT has been established as a powerful tool for studying nascent protein synthesis in a broad range of biological contexts. For example, the method has been used to study localized translation in mammalian neuronal cultures [3], newly synthesized proteins in mice [4], and to selectively target tissues in *Caenorhabditis elegans* [5], and *Drosophila melanogaster* [6]. In bacteria, time-selective labeling with Aha has revealed the dynamic proteome of *Bacillus subtilis* reviving from spores [7] and proteomics of the quorum sensing response of *Vibrio harveyi* [8], while cell-selective approaches have been used to

identify bacterial proteins important for host-cell infection [9, 10]. See Yuet and Tirrell for a comprehensive review [11].

Because BONCAT offers sensitive temporal and cellular selectivity, we thought it would be particularly well suited to address questions of bacterial slow growth and heterogeneity. Of particular interest was the opportunistic pathogen *Pseudomonas aeruginosa*, a gram-negative bacterium whose infections of the cystic fibrosis (CF) lung are chronic and recalcitrant to both the host immune system and to antimicrobial therapies. These infections are characterized by bacteria in a dormant state with low metabolic rates, a physiological condition known to contribute to antibiotic tolerance. Additionally, *P. aeruginosa* grows as biofilm microcolonies within the CF lung, a growth state in which nutrient gradients lead to phenotypic heterogeneities that contribute further to tolerance. We set out to learn more about the physiology of these states *in vitro* through selective proteomics. My contributions were twofold: (i) the application of BONCAT to bacterial systems in which low metabolic rates or cellular heterogeneity create difficulties for traditional proteomic analyses and (ii) the discovery and characterization of a new regulatory protein that helps *P. aeruginosa* to adapt to these challenging conditions.

Chapter 2 describes the application of the BONCAT method for temporally-selective proteomic analysis. In this work, we determined the nascent proteome of *P. aeruginosa* subsisting in an anoxic survival state. Analysis of proteins preferentially expressed in this state led to the discovery of a previously uncharacterized transcription factor, which we now call SutA (survival under transitions). We found SutA to be important for biofilm formation, the production of *P. aeruginosa*'s phenazine virulence factors, and the organism's ability to adapt to changing conditions. We identified an interaction between SutA and RNA polymerase, and through this interaction, its association with much of the chromosome. In particular, SutA shows high levels of association with loci encoding ribosomal components (ribosomal proteins and ribosomal RNA) and its presence in the cell enhances the expression of these genes. In addition, SutA generally shifts the gene expression profile away from genes involved in primary metabolism and toward those involved in cellular maintenance and secondary metabolisms.

Chapter 3 describes our investigations into the physical interaction between SutA and RNA polymerase. Because the primary amino acid sequence of SutA does not match any characterized proteins or domains, nothing was known about its mechanism of transcriptional regulation. We undertook a series of *in vitro* experiments to

characterize the structure of SutA and the nature of its binding to RNA polymerase. Through chemical cross-linking and protein foot-printing, we find evidence that SutA binds the β lobe 1 and β' clamp domains of RNA polymerase and, through this interaction, may elicit a conformational change of RNA polymerase. We suggest how this function may explain the physiological effects described in Chapter 2.

Chapter 4 describes the adaptation of the cell-selective BONCAT method for the study of heterogeneous *P. aeruginosa* biofilms. We take advantage of phenotypic differences between biofilm cells to restrict BONCAT labeling to an antibiotic-tolerant subpopulation. We characterize our ability to selectively enrich and identify proteins synthesized by this subpopoluation. We then determine the dynamic proteomic response of these cells to antibiotic challenge with the clinical antibiotic ciprofloxacin, and place the measured proteomic changes into biological context.

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