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Phenotypic knockouts of selected metabolic pathways by targeting enzymes with camel-derived nanobodies (V_{HH}s)

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ABSTRACT

Surveying the dynamics of metabolic networks of Gram-negative bacteria often requires the conditional shutdown of enzymatic activities once the corresponding proteins have been produced. We show that given biochemical functions can be entirely suppressed *in vivo* with camel antibodies (V_{HH}s, nanobodies) that target active sites of cognate enzymes expressed in the cytoplasm. As a proof of principle, we raised V_{HH}s against 2,5-dihydroxypyridine dioxygenase (NicX) of *Pseudomonas putida*, involved in nicotinic acid metabolism. Once fused to a thioredoxin domain, the corresponding nanobodies inhibited the enzyme both in *Escherichia coli* and in *P. putida* cells, which then accumulated the metabolic substrate of NicX. V_{HH}s were further engineered to track the antigen *in vivo* by C-terminal fusion to a fluorescent protein. Conditional expression of the resulting V_{HH}s allows simultaneously to track and target proteins of interest and enables the design of transient phenotypes without mutating the genetic complement of the bacteria under study.

1. Introduction

The customary approach for studying and eventually redesign metabolic pathways involves entering perturbations in specific nodes of otherwise balanced networks (Long and Antoniewicz, 2014). In practical terms, this is brought about by either mutating/deleting a selected site of the pathway (knock-out), by entering a new activity (knock-in) or by making the onset of a given activity dependent on an external signal (as is typical of inducible expression systems). Although conditional production of a new protein (or even a whole route) in an existing organism, *e.g.* a bacterium is easy to engineer, deliberate removal or inhibition of an ongoing enzymatic activity poses a more intricate technical challenge (Holtz and Keasling, 2010). Active proteins may remain in the bacterial cells for much longer than their mRNA has ceased to be produced or its translation has been inhibited with a riboswitch (Desai and Gallivan, 2004), small RNAs (Na et al., 2013) or siRNA in the bacterial cytoplasm (Man et al.), thereby letting the network to resettle and thus conceal the effects of the perturbation. Furthermore, the presence of many RNAses in different bacteria is often a bottleneck to engineer artificial RNA-based conditional expression systems (Conrad and Sonnewald, 2003). The issue is therefore how to inhibit specific biological functions while they are well in action, but keeping the integrity of the proteins involved. To this end, what could be the active agents for such a selective post-translational inhibition of enzymatic activity without destroying the proteins properly? On this background we entertained the possibility of using antibodies (AB) specific for a given enzymatic target and which could be expressed intracellularly for that purpose. Various types of recombinant ABs have been used in yeasts (Visintin et al., 1999), animal cells (Lo et al., 2008) and plant cells (Jobling et al., 2003) for controlling diverse intracellular activities. However, the same strategy has not found any significant application in prokaryotes due to the difficulty of folding the business part of the ABs (*e.g.* recombinant scF_v domains, which hold disulfide bonds) in the reducing milieu of the bacterial cytoplasm. Luckily, the last few years has witnessed different strategies for intracellularly producing active AB fragments in *Escherichia coli* and in other Gram-negative bacteria by either manipulating genetically the redox potential of the cytoplasm (Jurado et al., 2006b) or by fusing the AB fragment to a thioredoxin moiety (Zafra et al., 2011), respectively. These observations, however, have not been translated into a general stratagem for making post-translational activity knockouts.

The approaches presented below capitalize our previous research on intracellular expression of recombinant ABs (Jurado et al., 2006a) for enabling the functional suppression of selected metabolic pathways – but in a fashion that keeps intact the protein structure of the targeted enzyme. The strategy involves the

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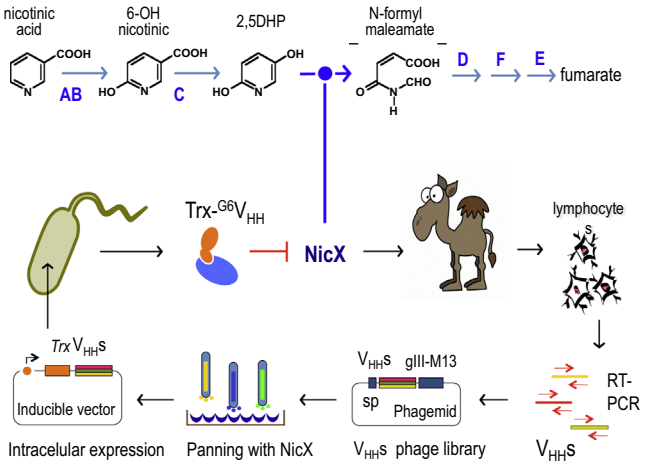

Fig. 1. Rationale of the strategy to generate phenotypic knockouts. In the presence of nicotinic acid, the pathway for its degradation will be activated (genes *nicA* to *F* shown in blue letters) and the compound transformed into several intermediates before entering the central metabolism *via* the tricarboxylic acids cycle. In that pathway NicX encodes the cleavage of 2,5DHP using oxygen as a co-substrate. The workflow to obtain dedicated V_{HH}s against the target enzyme is shown below and explained in the Supplementary methods. When a dedicated AB that inhibits NicX is expressed, the substrates and the AB compete for the active site of the enzyme that is eventually blocked by the latter. As a consequence, the substrate 2,5DHP is accumulated and the culture acquires a characteristic green phenotype. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

refactoring to this end of the distinct type of ABs produced by camelids *e.g. Camelus dromedarius.* The small (12–15 kDa) antigen-binding domains of such *camelbodies* (the V_{HH}s, called also *nanobodies* (Cortez-Retamozo et al., 2004)), are composed of just one polypeptide (Hamers-Casterman et al., 1993). This is in contrast with F_{ab}s of typical immunoglobulins or the artificially-linked two chains of scF_vs. In addition, they seem to have evolved to block active centers of enzymes (De Genst et al., 2006). Therefore, provided that V_{HH}s can be made specific for chosen enzymes and that they can be expressed intracellularly in an active form, such nanobodies appear as promising candidates to bring about the conditional phenotypic knockout of metabolic reactions envisioned above.

The work below accredits the feasibility of using V_{HH}s in metabolic engineering of Gram-negative bacteria by addressing, as a study case, the production of the valuable compound 2,5-dihydroxypyridine (2,5DHP) out of nicotinic acid (Jiménez et al., 2008) (Fig. 1). To this end we programmed the soil microorganism *Pseudomonas putida* KT2440 to express in its cytoplasm a nanobody from a camel immunized with purified NicX (2,5-dihydroxypyridine 5,6-dioxygenase, DHPDO), the key enzyme of the nicotinate degradation pathway of this bacterium (Fig. 1). *P. putida* KT2440 is growingly recognized as an optimal platform for hosting reactions that involve harsh redox biotransformations (Nikel et al., 2014) and therefore it is an ideal holder of the conversion nicotinate→2,5DHP (Fig. 1) pursued in this case.

Along the process, we found that [i] functional V_{HH}s can be generated and formatted for conditional and cytoplasmic expression in *P. putida* (and surely other Gram-negative bacteria), [ii] that the anti-NicX V_{HH} that best inhibited DHPDO activity did so by irreversibly blocking the entry of substrate in the active site of the enzyme and [iii] that NicX appears to be evenly distributed through the whole cytoplasm of *P. putida.* Unlike other strategies that require changes in genes and promoters of the genome of the host cells, the V_{HH}-based approach presented here endows bacteria with defined metabolic phenotypes without any modification of the host chromosome.

2. Results and discussion

2.1. Rationale of the enzyme track-and-target strategy

Bacterial activities on *N*-heterocyclic compounds are one of the major pursuits of contemporary metabolic engineering (Hawkins and Smolke, 2008; Keasling, 2008; Keasling, 2010). Some interesting enzymes to this end can be found, for instance, in a route in the soil bacterium *Pseudomonas putida* for mineralization of nicotinic acid (NA) an alkaloid included in the pyridine family (Behrman and Stanier, 1957). The key step in the pathway is the cleavage of the metabolite 2,5DHP carried out by the dioxygenase NicX using molecular oxygen as a co-substrate in the reaction and generating *N*-formylmaleamic acid (NFM) as a product (Fig. 1) (Jiménez et al., 2008). Generation of 2,5DHP is particularly appealing because it is used as a building block in the synthesis of pesticides and antitumoral drugs (Yoshida and Nagasawa, 2000). In addition, its accumulation generates a characteristic green phenotype that can be easily detected (Behrman and Stanier, 1957). *P. putida* KT2440 was thus chosen as a study case to explore the ability of intracellularly expressed nanobodies for selective blocking of intracellular metabolic pathways, specifically its chromosomally-encoded *nic* pathway for degradation of NA (Fig. 1). This strain has been widely used for industrial and environmental purposes because its diverse metabolism and ease of genetic manipulation (Nikel et al., 2014). The work below thus deals with the stratagem sketched in Fig. 1 to bring about *in vivo* inhibition of NicX in the cytoplasmic milieu of *P. putida* with an intracellularly expressed camel-derived ABs. Such AB fragments (nanobodies) consist of just one antigen-binding domain and have an improved solubility, robustness and ease of folding that facilitate their expression in bacteria – despite some caveats addressed below. But the first step was obviously the generation of highly specific V_{HH}s capable of not only strongly binding NicX, but also inhibiting its enzymatic activity.

2.2. Harvesting and selecting anti-NicX VHHs

To identify good NicX inhibitory nanobodies we run the process summarized in Fig. 1. For this we recovered lymphocytes from the blood of a specimen of *Camelus dromedarius* (Zafra et al., 2011) which had been inoculated (*inter-alia*) with the purified protein. The RNA was extracted, retrotranscribed and the DNA of the V_{HH} domains amplified, cloned as fusions to the pIII protein of M13 phage and the resulting display library panned twice for NicX binding as shown in the Section 3 (Zafra et al., 2011). 120 individual nanobody-displaying phage clones were separately tested in ELISA assays against NicX, thereby manifesting different binding affinities to the antigen (Suppl. Fig. 1). Approximatley 10^9 capsides of the six M13 clones giving the strongest ELISA signal were directly tested for *in vitro* inhibition of NicX activity as explained in the Section 3, causing inhibitions ranging from 15% to 80% of the activity of the enzyme with M13 phages not displaying a V_{HH} (not shown).

The DNA fragment encoding the V_{HH} with the highest inhibition rate when displayed in M13, named G6, was then selected for intracellular expression. To this end, the corresponding sequence was amplified by PCR and cloned in broad-host vector pBBR1●MCS5, resulting in plasmid pBG6 (Table S2). This construct expresses the $^{G6}V_{HH}$ DNA coding sequence as a single 127 amino acid polypeptide, transcribed under the control of a constitutive *lac* promoter (Fig. 2A). pBG6 was then passed to *P. putida* KT2440 and the resulting clones cultivated in minimal medium with nicotinate as sole C source. Should $^{G6}V_{HH}$ inhibit NicX activity *in vivo* (as it did *in vitro*), then cells may not grow in such a selective medium. The data of Fig. 2B, however, indicated this not

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