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A complete structural characterization of the desferrioxamine E biosynthetic pathway from the fire blight pathogen *Erwinia amylovora*

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ABSTRACT

The Gram-negative bacterium *Erwinia amylovora* is the etiological agent of fire blight, a devastating disease which affects Rosaceae such as apple, pear and quince. The siderophore desferrioxamine E plays an important role in bacterial pathogenesis by scavenging iron from the host. DfoJ, DfoA and DfoC are the enzymes responsible for desferrioxamine production starting from lysine. We have determined the crystal structures of each enzyme in the desferrioxamine E pathway and demonstrate that the biosynthesis involves the concerted action of DfoJ, followed by DfoA and lastly DfoC. These data provide the first crystal structures of a Group II pyridoxal-dependent lysine decarboxylase, a cadaverine monooxygenase and a desferrioxamine synthetase.

DfoJ is a homodimer made up of three domains. Each monomer contributes to the completion of the active site, which is positioned at the dimer interface. DfoA is the first structure of a cadaverine monooxygenase. It forms homotetramers whose subunits are built by two domains: one for FAD and one for $NADP^+$ binding, the latter of which is formed by two subdomains. We propose a model for substrate binding and the role of residues 43–47 as gate keepers for FAD binding and the role of Arg97 in cofactors turnover. DfoC is the first structure of a desferrioxamine synthetase and the first of a multi-enzyme siderophore synthetase coupling an acyltransferase domain with a Non-Ribosomal Peptide Synthetase (NRPS)-Independent Siderophore domain (NIS).

1. Introduction

Fire blight is caused by the Gram negative bacterium *Erwinia amylovora*, which is considered by Mansfield et al. to be one of the top ten known plant pathogens due to its potential impact to the global apple and pear production (Vanneste. 2000,Mansfield et al. 2012). When environmental conditions favor infection, a fire blight outbreak can cause the loss of entire annual harvests and lead to dramatic economic damages. For example, in the year 2000, Michigan's economy lost $42 million as a direct result of a fire blight outbreak (Norelli et al. 2003). Currently, the control methods are quarantine, pruning and/or eradication of the infected plants, the use of biological and chemical pesticides, antibiotics, and resistant cultivars obtained by classical breeding or by genetic engineering (Gusberti et al. 2015). However, the use of antibiotics and genetically modified plants are not allowed in most countries. Therefore, understanding the structure and function of *E. amylovora* enzymes, essential for infectivity/pathogenicity, is strategically relevant, as the acquired knowledge can contribute to the potential development of novel control measures.

Three main molecular systems are required for a successful infection in *E. amylovora*: the type three secretion system (T3SS), the exopolysaccharide biosynthetic pathway, and iron uptake (Vrancken et al. 2013,Piqué et al. 2015,Expert et al. 2000). The T3SS is responsible for secretion of effectors into the host plant, while the exopolysaccharide is used by the bacterium for biofilm formation and protection against the host defense mechanism. In *E. amylovora* CFBP1430 Dellagi et al. observed that mutants, obtained by insertional mutagenesis, defective in the siderophore biosynthesis (disrupted *dfoA* gene) and siderophore

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uptake (ferrioxamine receptor FoxR defective) showed a two orders of magnitude reduced growth on apple flowers compared to the wild-type strain (Dellagi et al. 1998). In this work, we focus on the enzymes involved in *E. amylovora* siderophore biosynthesis. An effective iron uptake system is important as iron is an essential nutrient used as a protein cofactor in almost every living organism. Iron bioavailability for plant pathogenic bacteria is very low in the pathogen hosts. In fact, iron homeostasis is very strict and the free iron is kept to $\sim 10^{-24}$ M. In nature, iron is found mainly as Fe(II) or Fe(III). Fe(II) is soluble and can be internalized by specific divalent metal transporters (Miethke and Marahiel. 2007,Johnstone and Nolan. 2015), but it is very prone to oxidation to Fe(III), that in turn forms very stable ferric oxide-hydrate complexes, which are insoluble at neutral to alkaline pH and aerobic conditions. In order to solubilize and acquire iron from their environment, living organisms have developed different methods. Direct iron uptake depends on specific receptors (*e.g.* receptors for transferrin, ferritin, heme, hemoproteins) (Miethke and Marahiel. 2007), while indirect uptake relies on hemophores or siderophores. In particular, siderophores are among the strongest Fe(III) chelators and the most widespread and successful biological iron scavengers known (Li et al. 2016). Siderophores are usually low molecular weight molecules (400–1000 Da) secreted by the cell for ferric iron complexation and reinternalized via specific receptors (Koster. 1991). In *E. amylovora* siderophore mediated iron uptake is dependent on desferrioxamines (DFOs). At least 20 different types of microbial DFOs exist (http://bertrandsamuel.free.fr/siderophore_base/siderophores.php?find=D). They consist of diamine and dicarboxylic acid building blocks linked by amide bonds. The major product of DFO biosynthesis in *E. amylovora* is desferrioxamine E (DFO-E; nocardamine), while other DFOs (D2, X1-7 and G1-2) are produced in lower quantities (Feistner et al. 1993,Feistner. 1995,Kachadourian et al. 1996). Desferrioxamines are very efficient as they are among the strongest hydroxamate siderophores known. For example, DFO-E has an affinity constant for Fe(III) of 32.5 ($\mathrm{Log}K_f$ Fe(III)) (Hider and Kong. 2010).

In *E. amylovora*, DFO-E has different roles. The main role is to overcome iron starvation (Dellagi et al. 1998,Johnstone and Nolan. 2015). Additionally, DFO-E is used as a protection against the infection triggered oxidative burst and could also act as a substrate of plant cell wall peroxidases in the presence of H_2O_2 to afford radical nitroxides, enhancing oxidative stress induced by hairpins and resulting in an overall increase of electrolyte leakage from the host plant (Dellagi et al. 1998).

In *E. amylovora* a single gene cluster encodes for three proteins, DfoJ, DfoA and DfoC, responsible for the biosynthesis of DFO-E (*dfoJAC* operon) (Smits and Duffy. 2011). These proteins perform the four enzymatic reactions presented in Fig. 1. The overall reaction stoichiometry is as follows:

$$3Lysine + 3SuccinylCoA + 3O_2 + 3NADPH + 3H^+ + 3NTP = DFO + 3CoA + 3CO_2 + 6H_2O + 3NADP^+ + 3NMP + 3PP_i \quad (1)$$

In *S. coelicolor A3(2)*, a cluster of four genes (desA-D), analogous to *dfoJAC* operon, has been identified (Barona-Gomez et al. 2004). *S. coelicolor A3(2)* has two distinct structural genes instead of the single DfoC gene alone (Barona-Gomez et al. 2004). Therefore, DfoC may derive from an evolutionary gene fusion process. The presence of the acyltransferase and the siderophore synthetase in the same protein could be justified by the fact that the acyltransferase product, N-5-aminopentyl-N-(hydroxyl)-succinamic acid, is chemically unstable and the simultaneous presence of both enzymatic activity would favor siderophore ring formation. It has been speculated that the reaction pathway could be identical to that of *Streptomyces coelicolor* (Fig. 1, following the thicker arrows) (Challis. 2005,Smits et al. 2011). Nevertheless, there is a potential alternative way analogous to the aerobactin biosynthetic pathway (Fig. 1, following the thinner arrows for the DfoJ and DfoA reactions). DfoA is a cadaverine monooxygenase but is currently annotated as L-lysine N6-monooxygenase (EC 1.14.13.59), as it would be in the alternative pathway. On the other hand, there is experimental evidence for the pathway to start with an initial lysine decarboxylation, but no evidence for an initial lysine monooxygenation (Feistner. 1995,Expert et al. 2000). Feistner characterized DFO production by *E. amylovora* EA1430 and discovered that the main product incorporated cadaverine (group 1) but other DFOs could contain, together with cadaverine, ornithine (group2) or cadaverine, ornithine and diaminopropane (group 3) suggesting that the activity of the enzymes of the pathway could be carried out on different substrates. By feeding either diaminohexane or 2-hydroxyputrescine to bacterial cultures, DFOs containing one moiety each of these compounds together with two moieties of cadaverine were obtained (Feistner. 1995). To date *in vitro* substrate specificity of the enzymes of the DFOs pathway has never been characterized despite it relevance.

We have determined the structures of DfoJ, a Group II pyridoxal-dependent lysine decarboxylase, DfoA, a cadaverine monooxygenase and DfoC, a desferrioxamine synthetase.

The structural data provide insight into the biosynthetic pathway of desferrioxamine E in *E. amylovora* and in particular the role of DfoA and the order of the initial enzymatic steps of the pathway.

2. Materials and methods

Chemicals were bought from SIGMA Aldrich, unless otherwise stated.

2.1. Gene cloning and protein expression

DfoJ (UniProt accession No. D4I245_ERWAC; Ordered Locus Name No. EAMY_3238; EC: 4.1.1.18), DfoA (UniProt accession No. D4I246_ERWAC; Ordered Locus Name No. EAMY_3239; EC: 1.14.13.-) and DfoC (UniProt accession no D4I247_ERWAC; Ordered Locus Name No. EAMY_3240; EC: 2.3.-.-, 6.3.-.-) from *E. amylovora* (TaxID No. 665,029) were amplified by PCR from genomic DNA (strain CFBP1430) (Table S1). The PCR products were digested by restriction enzymes (Table S1) and cloned into a pETM11 (EMBL) vector that fuses a His_{6x}-tag and a TEV protease cleavage site at the protein N-terminus (Dümmler et al. 2005). The obtained constructs were used to transform a BL21(DE3)Star-pLysS *E. coli* strain in case of DfoJ and DfoC and a BL21(DE3) *E. coli* strain in the case of DfoA. Cells were grown at 310 K, 220 rpm in auto inducing medium (Studier. 2005) until an OD_{600} between 2 and 2.5. Then, the cells were chilled on ice for 15′ and the expression was carried out at 293 K, 220 rpm for 16 h. The sequences of the expressed proteins are presented in Table S2. Cells were harvested by centrifugation and resuspended in 1/20 culture volume of 50 mM PBS pH 7.4 (pellet wash step) before centrifuging a final time.

2.2. DfoJ purification

The washed cells pellet was centrifuged and resuspended in 1/50 culture volume of lysis buffer: 50 mM PBS pH 7.5, 250 mM NaCl, 20 mM Imidazole, 0.5 mM TCEP, 0.25 mg ml^{-1} lysozyme, one protease inhibitor cocktail tablet (Roche, Swiss). Cells were disrupted by sonication and the lysate was clarified by centrifugation and filtration. The first purification step consisted of an immobilized metal affinity chromatography (IMAC). A 5 ml HisTrap Chelating column (GE Healthcare) was equilibrated with 50 mM PBS pH 8, 250 mM NaCl, 20 mM Imidazole, 0.5 mM TCEP (buffer A). The protein was bound to the column. Unbound sample was washed out with the same buffer used for equilibration. More impurities were washed out with a 90 mM imidazole buffer obtained mixing buffer A and buffer B (50 mM PBS pH 8, 250 mM NaCl, 500 mM Imidazole, 0.5 mM TCEP). DfoJ was eluted at 200 mM imidazole. The eluted protein solution appeared yellow because of Schiff's base formation in a pyridoxal phosphate (PLP)-bound enzyme (Eliot and Kirsch. 2004). The Protein was concentrated using a