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High-yield production of a functional bacteriophage lysin with antipneumococcal activity using a plant virus-based expression system

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ABSTRACT

Streptococcus pneumoniae is the causative agent of several serious infectious diseases. It is becoming increasingly antibiotic resistant worldwide, and thus new antimicrobials are needed. One alternative to antibiotics may be the use of peptidoglycan hydrolases, the bacteriophage lytic enzymes.

In this study, we demonstrated high level expression of the *S. pneumoniae* bacteriophage lysin Pal in *Nicotiana benthamiana* – TMV (Tobacco Mosaic Virus) transient expression system. The protein was purified to homogeneity and tested for streptococci killing activity *in vitro* and *in vivo*. *In vitro*, Pal was able to lyse three tested *S. pneumoniae* strains: NCTC12695, NCTC12977 and NCTC11888. The treatment of BALB/c mice with 100 μg, 200 μg and 400 μg of Pal 1 h post-challenge with double lethal dose of *S. pneumoniae* NCTC12695 strain showed a clear dose response and protected from lethal sepsis 30%, 40% and 50% of mice, respectively. The improved mice survival correlated with decreased blood bacterial titers. In conclusion, these results suggest that plant-expressed bacteriophage lysins may have potential use as antimicrobial agents.

1. Introduction

Streptococcus pneumoniae is the cause of various severe infectious diseases such as otitis media, rhinosinusitis, bronchitis, pneumonia, endocarditis, meningitis and sepsis. Young children and the elderly are the most susceptible age groups. The resistance of pneumococci to a variety of antimicrobial agents has evolved to a worldwide health problem. Pneumococcal resistance has increased to a point that it is clinically relevant in several classes of antibiotics: beta-lactams, macrolides, tetracyclines and folate inhibitors. Multi-resistant pneumococci are being encountered more commonly and are more often community acquired (Klugman, 1990). Thus, new antimicrobial agents are needed to counteract the growing problem of antibiotic resistance.

Bacteriophage lysins are extensively studied lately for their potential to be used instead of antibiotics or to enhance the efficiency of antibiotics. Bacteriophage lysins destroy the peptidoglycans to release the virion progeny from phage-infected host bacteria (Young, 1992). Purified lysins have shown very promising potential for the treatment of infectious diseases caused by Gram-positive bacteria (Fischetti, 2008). Bacteriophage lysins have been shown to achieve their antibacterial action when added externally to the target bacteria and were very potent in various *in vivo* models of infection (Loeffler et al., 2001). The absence of reports on phage endolysin-resistant bacterial strains can be considered as an advantage of using a lysin-based approach to fight bacterial infections. A likely explanation for the absence of observed resistance to endolysins is that the bacterial host and phage have coevolved in such way that phage endolysins target immutable bonds in order to ensure phage survival and release from the host (Fischetti et al., 2006; Nelson et al., 2012).

A large panel of bacteriophage lysins specific to gram positive and even gram negative microbial pathogens has been cloned and tested for their activity *in vitro* and *in vivo*. Probably, the most

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studied endolysins in animal models are Cpl-1, an N-acetylmuramidase from the Cp-1 pneumococcal phage, and Pal, an N-acetylmuramoyl-L-alanine amidase from the DP-1 pneumococcal phage (Nelson et al., 2012). Cpl-1, the lysozyme from bacteriophage Cp-1 (García et al., 1987), has been successfully tested in animal models reproducing several human pneumococcal diseases such as bacteremia (Jado et al., 2003; Loeffler et al., 2003), pneumonia (Witzenrath et al., 2009; Doehn et al., 2013), upper respiratory colonization (Nelson et al., 2001), otitis media (McCullers et al., 2007), endocarditis (Entenza et al., 2005) and meningitis (Grandgirard et al., 2008). Pal (Sheehan et al., 1997) has been shown to efficiently eradicate pneumococci in a mouse model of nasopharyngeal carriage (Loeffler et al., 2001) and to act synergistically with Cpl-1 *in vitro* (Loeffler and Fischetti, 2003) and in *vivo* (Jado et al., 2003).

All studies described above were accomplished with *Escherichia coli*-expressed lysins. Alternatively, successful expression of bacteriophage Cpl-1 and Pal lysins has been achieved also in transplastomic plants (Oey et al., 2009). Both lysins have shown good levels of expression in tobacco chloroplasts and were active *in vitro* against *S. pneumoniae*, but no *in vivo* experiments have been described.

The production of recombinant proteins in plants presents several advantages over the commonly used expression systems based on animal cells, yeast or bacteria because of lower production costs, easy scale-up and reduced risks of pathogen contamination. However, the generation of transgenic and transplastomic plants is laborious and time consuming. Plant virus-based expression systems, in which the foreign mRNA is amplified by the replicating virus, can produce very high levels of proteins in leaves and other tissues. Plant virus vectors have been developed as an alternative to stably transformed transgenic or transplastomic plants because of several advantages such as expression speed and yield, reduced cost/duration of research and development and very high throughput (Gleba and Giritch, 2011).

This study was designed to test the suitability of the plant virus-based expression system for the production of functional bacteriophage lysins. We describe here the high level expression of *S. pneumoniae* bacteriophage lysin Pal in a *Nicotiana benthamiana* – TMV transient expression system, followed by protein purification and lysin activity studies *in vitro* and in mice bacteremia model.

2. Materials and methods

2.1. Construction of Pal expression plasmids

The coding sequence of Pal from phage DP-1 (ACB88203) was codon-optimized for *N. benthamiana* and synthesized by Entelechon GmbH (Bad Abbach, Germany). The sequence was inserted as a *BsaI-BsaI* fragment into the pICH31070 Δ lacZ plasmid (MagnICON deconstructed tobacco mosaic virus (TMV) system 3′ provector (Marillonnet et al., 2004)) for expression studies, and into the pICH29912 (assembled TMV-based MagnICON vector (Marillonnet et al., 2005)) for large scale expression. The constructs obtained were named pNMDV132 (pICH31070-Pal) and pNMD2671 (pICH29912-Pal) (Fig. 1). pNMDV132 and pNMD2671 vectors were used to transform *Agrobacterium tumefaciens* strain GV3101.

2.2. Pal transient expression in plants

N. benthamiana plants were grown in a growth chamber at 25 °C with a 16 h light and 8 h dark photoperiod. Five-six-week-old plants were used for infiltration and for spraying with recombinant *A. tumefaciens*.

A. tumefaciens were grown overnight at 30 °C in Luria–Bertani media containing 50 mg/l rifampicin and other appropriate antibiotics depending on the type of plasmids (50 mg/l kanamycin for selection of integrase, TMV 3′ provector and TMV assembled vector; 50 mg/l carbenicilin for selection of 5′ TMV provectors). *Agrobacterium* overnight cultures were adjusted to an OD_{600} of 1.5, sedimented at 3220 × *g* for 5 min and resuspended in equal volume of infiltration buffer (10 mM MES pH 5.5, 10 mM $MgSO_4$).

2.2.1. Syringe infiltration of N. benthamiana leaves

A mix of three *A. tumefaciens* strains containing the 5′ provector, 3′ provector and integrase plasmid constructs was infiltrated into the abaxial side of the leaf using a syringe without a needle. MagnICON 5′ provector modules for apoplast targeting (pICH20155 and pICH22464, with His-tag), cytosol targeting (pICH20111 and pICH22455, with His-tag) and chloroplast targeting (pICH20030 and pICH22474, with His-tag) as well as the integrase expression vector pICH14011 were used together with the pNMDV132 3′ module. Strains containing 5′ and 3′ provector modules were used at dilution 1:100, and the integrase cassette-containing strain at dilution 1:40. Plant leaves were observed and collected at five-six days post infiltration. pICH20155, pICH22464, pICH20111, pICH22455, pICH20030, pICH22474, pICH7410 and pICH14011 constructs are described in Kalthoff et al. (2010).

2.2.2. Spraying of N. benthamiana leaves

For larger scale Pal expression and purification, *N. benthamiana* plant leaves were sprayed with a 1:1000 dilution of *A. tumefaciens* strain containing pNMD2671 vector as described in Hahn et al. (2014). Spraying was performed using infiltration buffer containing 0.1% (v/v) Silwet L77 (Kurt Obermeier GmbH & Co. KG, Bad Berleburg, Germany). Plant leaves were observed and collected at six days post spraying.

2.3. Pal extraction and purification from N. benthamiana plants

Plant material was homogenized with chilled mortar and pestle and mixed with lysis buffer (50 mM HEPES-KOH, 10 mM KAc, 5 mM MgAc, 5 mM β-mercaptoethanol, 300 mM NaCl, 0.05% Tween 20, 10% glycerol, pH 7) at a ratio of 1 g of tissue to 5 ml of buffer. Cell debris were removed by centrifugation at 3220 × *g*, at 4 °C for 60 min. The supernatant was filtered and taken as total soluble protein.

Pal protein was purified by affinity chromatography with ÄKTA™purifier (GE Healthcare). The total soluble protein was applied to a DEAE Sepharose™ Fast Flow (GE Healthcare) column (Pharmacia C 10/10). 300 ml of crude protein extract was applied at flow rate 2 ml/min. The column was washed at flow rate 2 ml/min with 100 ml of wash buffer (50 mM HEPES-KOH, 10 mM KAc, 5 mM MgAc, 1.5 M NaCl, 0.05% Tween 20, 10% glycerol, pH 6.9) until no protein was detected in the eluate. Elution at flow rate 0.5–1 ml/min was performed with the same buffer containing 2% choline chloride, pH 6.9. UNICORN 5.2 (Build 500) software was used for data evaluation. Purified Pal was dialyzed against dialysis buffer (50 mM of sodium phosphate buffer, pH 7, 1 mM DTT, 1 mM EDTA), filter-sterilized (0.2 μm), lyophilized and stored at −20 °C.

2.4. Pal bacteriolytic activity against S. pneumoniae strains

To evaluate Pal influence on colony counts of *S. pneumoniae*, NCTC12977 strain was inoculated overnight in THY media containing 0.1% of choline chloride and grown in CO_2-enriched atmosphere to an OD_{600} of 0.6. The cells were washed and resuspended in PBS pH 7 to a final OD_{600} of 1. 800 μl of *S. pneumoniae* suspension were mixed with 2.5 μg of purified dialyzed Pal in 200 μl