# Improved Lethal Effect of a Phage Pneumococcal Lysozyme by Changing the Net Charge

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**ABSTRACT:**

Bacteriophage lytic murein-hydrolases have been proposed as an efficient way to fight bacterial infections. However, the use of these enzymes is normally restricted to Gram-positive bacteria since the outer membrane of the Gram-negative bacteria hampers the access of the protein to its peptidoglycan substrate. All the murein hydrolases reported in the pneumococcal system, both from host or phage origin, depend on the aminoalcohol choline to be fully active. There is only a unique exception to this rule, the Cpl-7 lysozyme, encoded by the lytic pneumococcal phage Cp-7, which instead of the common cell wall binding module recognizing choline, harbors a completely different cell wall module. Studies developed in our laboratories have allowed the improvement of Cpl-7 antibacterial activity by reducing the net charge from -28.8 to -10.85 introducing 15 amino acid substitutions in the cell wall binding module. This modified enzyme, Cpl-7S, was capable to lyse not only the pneumococcal strains tested, including the antibiotic-multiresistant D48 strain, but also a variety of Gram-positive bacteria. We have also designed a standard protocol to destabilize the outer membrane of Gram-negative bacteria and turning them susceptible to the action of Cpl-7S.

In this communication, we will present the results of Cpl-7S, which constitutes a promising alternative to kill not only pneumococcal cells but also other important pathogens like *Streptococcus pyogenes*, and even Gram-negative bacteria after sensitization of the outer membrane. This improved lysozyme, Cpl-7S, has also been tested in zebra fish embryos and conclusions drawn from this new animal model will be discussed.

**Keywords:** lysozyme, enzybiotic, phage, lysis.

## 1. Introduction

Currently, pneumococcal bacteriophage endolysins represent a promising weapon to be used as antimicrobial agents (enzybiotics) due to their high target cell specificity. Endolysins, or murein hydrolases, are classi-fied according to the different bonds cleaved in the peptidoglycan (PG): β-D-N-acetylmuramidases or lysozymes, β-D-N-acetylglucosaminidases, lytic transglycosy-lases, N-acetylmuramoyl–L-alanine ami-dases, and endopeptidases [1, 2].

Endolysins from phages infecting Gram-positive bacteria show a modular architecture, consisting of, at least, one catalytic domain that hydrolyzes a specific bond in the PG, and one cell wall-binding domain, which target the enzyme to its substrate [1]. It is noteworthy that all the murein hydrolases encoded by *S. pneumoniae* and its bacterio-phages are choline binding proteins (CBPs) with the noticeable exception of Cpl-7, a lysozyme encoded by the phage Cp-7 [2]. Cpl-7 lysozyme (muramidase class GH25) contains a C-terminal module completely different to the common choline binding module of CBPs and, thus, could act with a wider host range than the lytic CBPs [3].

We have found that Cpl-7 has lower enzymatic activity than Cpl-1, a choline-dependent phage lysozyme, in *in vitro* assays against pneumococcal cells. Since it has been documented that the net charge of the catalytic unit in this kind of enzymes is very important for the PG cleavage selectivity when acting from outside [4], we constructed a variant, designated as Cpl-7S, with five amino acid changes in each repeat of the cell wall binding module instead of modifying the catalytic module. In this communication, we will present the *in vitro* and *in vivo* experiments carried out with Cpl-7S.

## 2. Results and Discussion

### 2.1. Design of Cpl-7S

Based on the previous structural and func-tional studies of Cpl-7 [3, 5], we changed 15 non-conserved amino acids in the cell wall binding module, substituting acid or neutral amino acids by basic ones turning the net charge from –28.8 (Cpl-7) to –10.85 (Cpl-7S).

### 2.2. Production and purification of Cpl-7S

Gene fragment encoding Cpl-7S was synthetized in a pUC derivative plasmid, and sub cloned in a pT7-7 plasmid for overexpres-sion of the protein. Cpl-7S was purified in two chromatographic steps.

### 2.3. *In vitro* killing assay

Different Gram-positive bacteria were resus-pended in PBS buffer, pH 6.0, adjusted to an OD550 ≅ 0.6 and incubated at 37ºC for 60 min, with or without the lytic enzyme, added at 5 µg/ml. For Gram-negative bacteria, an additional destabilization of the outer membrane was necessary to facilitate the access of the enzyme to the substrate (PG). This can be achieved with 0.01% carvacrol, an essential oil, added in the PBS buffer (6). Evolu-tion of OD550 was followed and viable cells were measured after 15 and 60 min of incubation.

Table 1 summarizes the results obtained with Cpl-7S, and Cpl-1 and Cpl-7 as comparison, against a variety of bacteria tested after 60 min of incubation following the standard assay described above.

Taken together all the data, we can conclude that Cpl-7S is capable to recognize and hydrolyze the cell walls of several bacteria, broadening the host range of any pneumococcal murein hydrolase tested up to now. The bactericidal effect is seen in all the pneumococcal strains checked, including the antibiotic-multiresistant D48. Besides, Cpl-7S has a higher enzymatic activity than its parental variant Cpl-7, as the result of changing the net charge on the cell wall binding module.

We have validated the *in vitro* results of Cpl-7S with a new animal model, i.e., zebra fish embryos, whose conclusions will be presented.

*Table 1: Analysis of viable cells of the different strains studied.*

| Bacteria | Cpl-1 | Cpl-7 | Cpl-7S |
|---|---|---|---|
| Gram-positive | | | |
| *S. pneumoniae* | | | |
| R6 | * | +++ | ++++ |
| D39 | * | ++ | +++ |
| M11S3⁺ | * | ++ | ++ |
| M11S4⁺ | * | ++ | +++ |
| D48 | * | + | ++ |
| *S. pyogenes* | – | +++ | +++ |
| *S. mitis* SK598 | – | ++ | ++ |
| *S. agalactiae* | – | – | – |
| *S. iniae* | – | + | + |
| *S. mutans* | – | – | – |
| *S. dysgalactiae* | – | + | + |
| *S. aureus* | – | – | – |
| *B. adolescentis* | – | + | + |
| *C. jeikeium* | – | – | – |
| *L. lactis* | – | – | – |
| *M. smegmatis mc$^2$155* | – | – | – |
| Gram-negative | | | |
| *E. coli* | – | + | +++ |
| *P. putida* KT2442 | – | – | +++ |

(–) No effect, (+) decrease 1 log in viable cells, (+ +) decrease 2 logs in viable cells, (+ + +) decrease 3 log units in viable cells, (+ + + +) decrease 4 or more log units in viable cells; (*) <10 CFU / ml.

## 3. Conclusion

Pneumococcal phage lysins have been de-monstrated as powerful antimicrobials to fight

pneumococcal infections. With the use of Cpl-7S, we have extended the bacterici-dal effect to other Gram-positive and Gram-negative bacteria.

A detailed knowledge of structure and fun-ction of the Cpl-7 lysozyme has allowed the design of specific amino acid substitutions leading to an enzymatically improved va-riant of the enzyme (Cpl-7S) when added from outside.

*Acknowledgements*

This work was supported by grant SAF2009-10824 from Dirección General de Investigación Científica y Técnica, and Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES) (http://www.ciberes.org/), an initiative of the ISCIII.

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# Implication of diversity in the blp locus: distribution and phenotype of bacteriocin producers and non-producers

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## 1. Background

The *blp* locus contains the genes required for bacteriocin production in *S. pneumoniae*. The locus is controlled by the extracellular accumulation of one of four peptide pheromones (BlpC) through interaction with a dedicated two component regulatory system (BlpRH). Both BlpC and the functional bacteriocins are processed and secreted by the ABC transporter, BlpAB. Genes encoding a variety of bacteriocin and immunity proteins are found in the BIR (Bacteriocin/Immunity Region). Previous data demonstrated that nearly half of strains have a conserved 4bp insertion in the *blpA* gene which results in a non-functional transporter. These strains are unable to secrete BlpC or bacteriocins but retain the ability to respond to exogenous pheromone with production of bacteriocin specific immunity.

## 2. Methods

In this work, we examine the composition of the *blp* locus in 230 whole genome sequences (WGS) and correlate the findings with the phenotypes observed in a large strain collection. Sequences were used to evaluate linkage between pherotype and the integrity of the *blpA* ORF and to determine the spectrum of bacteriocin/immunity content. To demonstrate *in vitro* phenotypes in the population, overlay assays were performed on a total of 441 invasive and colonizing isolates derived from three separate collections. Pneumococci and related gram positive organisms were used to assess inhibitory activity. BlpC secretion was detected using pheromone specific reporter strains. BIR content in pheromone secreting strains was determined by RFLP analysis of the region and comparison with known loci. Deletions in bacteriocin encoding genes were created in a subset of inhibitory strains to determine the role of specific bacteriocins in inhibition.

## 3. Results

All 230 WGS contained a blp locus. The frame shifting 4bp repeat in *blpA* was identified in 51% of strains; 18% of strains had other frame shifts or deletions in *blpA*. *blpH* alleles corresponding to the four pherotypes were relatively evenly distributed, however, certain *blpH* alleles were more likely to be associated with a disrupted blpA gene than others. Only 8% of the $blpH_{T4}$ allele containing strains would have the capacity to secrete $BlpC_{T4}$ compared with 33-77% of the strains with the other *blpH* alleles. From the strain collections tested, *in vitro* evidence of *BlpC* secretion could be identified in 7-37% of isolates, similar to the numbers predicted from WGS analysis. We found that strains secreting $BlpC_{6A}$ could stimulate a $blpH_{T4}$ specific reporter, perhaps explaining the persistence of the $blpH_{T4}$ allele despite the near absence of $BlpC_{T4}$ secreting strains in the population. No other evidence of pheromone cross talk was noted. The BIR region of the WGS strains could be separated into 26 groups based on gene content. Members of a group often varied with respect to *blpH* allele and the integrity of *blpA*. WGS strains with identical sequence types by MLST were also variable in *blpA*/*H* content. *In vitro* inhibition was noted in 32% of pheromone secretors. Targeted mutagenesis demonstrated that the bacteriocins BlpIJ, BlpK, and $BlpMN_{6A}$ were associated with an inhibitory phenotype.