Microbiology Papers in Press. Published July 14, 2014 as doi:10.1099/mic.0.079111-0

Bacteriophage derived CHAP domain protein, P128, kills *Staphylococcus* cells by cleaving interpeptide cross bridge of peptidoglycan

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Keywords: MRSA, P128 protein, avirulent, peptidoglycan, resistant mutants, *femA*, pentaglycine

Running title: P128 acts on *S. aureus* pentaglycine cross bridge

Category: Cell and Molecular Biology of Microbes

Total number of words in the manuscript (including main text, tables and figure legends) – 7376

Total number of figures – 5; **Total number of tables** - 2

Abstract

P128 is an anti-staphylococcal protein consisting of *Staphylococcus aureus* phage K derived tail associated muralytic enzyme (TAME) catalytic domain (Lys16) fused with cell wall binding SH3b domain of lysostaphin. In order to understand the mechanism of action and emergence of resistance to P128, we isolated mutants of *Staphylococcus* spp, including MRSA, resistant to P128. In addition to P128, the mutants also showed resistance to Lys16, the catalytic domain of P128. The mutants showed loss of fitness as shown by reduced rate of growth *in vitro*. One of the mutants tested was found to be reduced in virulence in animal model of *S. aureus* septicemia suggesting loss of fitness *in vivo* as well. Analysis of the antibiotic sensitivity pattern showed that the mutants derived from MRSA strains had become sensitive to methicillin and other β-lactams. Interestingly, the mutant cells were resistant to lytic action of phage K, though the phage was able to adsorb to these cells. Sequencing of *femA* gene of three P128 resistant mutants showed either a truncation or deletion in *femA*, suggesting that improper cross bridge formation in *S. aureus* could be causing resistance to P128. Using GST fusion peptides as substrates it was found that both P128 and Lys16 were capable of cleaving a pentaglycine sequence, suggesting that P128 might be killing *S. aureus* by cleaving pentaglycine cross bridge of peptidoglycan. Moreover, peptides corresponding to reported cross-bridge of *S. haemolyticus* (GGSGG, AGSGG) which were not cleaved by lysostaphin, were cleaved efficiently by P128. This was also reflected in high sensitivity of *S. haemolyticus* to P128. This showed that in spite of sharing a common mechanism of action with lysostaphin, P128 has unique properties which allow it to act on certain lysostaphin resistant *Staphylococcus* strains.

Introduction

Staphylococcus aureus causes a variety of community acquired and hospital acquired infections in humans. Antibiotic resistance is quite common in this organism and emergence of hospital and community associated methicillin resistant *S. aureus* (MRSA) has worsened the situation further (David and Daum, 2010). Resistance has also been reported against latest drugs used for treating *S. aureus* such as vancomycin, daptomycin and linezolid (Kelley *et al*, 2011; Gu *et al*, 2013). Thus a persistent effort is required to discover new therapies against this important human pathogen.

Peptidoglycan hydrolases or Enzybiotics derived from bacteria or phages have been shown to possess potent anti-bacterial activities especially against Gram positive pathogens (Fenton *et al*, 2010; Rodríguez-Rubio *et al*, 2013; Szweda *et al*, 2012). Based on their ability to cleave specific bonds in the peptidoglycan, the murein hydrolases are classified as glycosidases, amidases or endopeptidases (Fenton *et al*, 2010; Szweda *et al*, 2012). The disruption of peptidoglycan results in leakage of cytoplasmic contents leading to cell death (Simmonds *et al*, 1996). Amongst the phage derived Enzybiotics, endolysins have received maximum attention and some of them have been shown to be efficacious in animal models of bacterial infections (Schmelcher *et al* 2012; Gilmer *et al*, 2013). Another category of murein hydrolases possessing potent antibacterial activity are the bacteriophage tail associated muralytic enzymes (TAMEs) (Paul *et a*l, 2011). In the phage life cycle TAMEs are involved in puncturing the bacterial cell wall for facilitation of phage DNA injection into the bacterial cytoplasm (Nakagawa *et al*, 1985). Since the TAME substrate (peptidoglycan) is conserved in a bacterial species, these enzymes have a broader spectrum of antibacterial activity compared to the narrower host range of bacteriophages (Paul *et al*, 2011; Narasimhaiah *et al*, 2013). Attempts to isolate bacterial mutants resistant to the

bactericidal action of muralytic endolysins have been unsuccessful (Loeffler *et al*, 2001; Schuch *et al*, 2002; Gilmer *et al*, 2013; Rodríguez-Rubio *et al*, 2013a). This has been attributed to the essential nature of the targets of endolysins and the inability of bacteria to tolerate any changes in these structures. On the other hand *S. aureus* mutants resistant to lysostaphin, a muralytic endopeptidase acting on pentaglycine cross bridge, could be isolated at high frequency (Climo *et al*, 2001). The mutations were mapped to *femA* gene which codes for pentaglycine synthase involved in addition of 2^{nd} and 3^{rd} glycine of the pentaglycine bridge in *S. aureus* peptidoglycan. *S. aureus* strains carrying mutant copies of *femA* were shown to possess single glycine residue in place of pentaglycine in the cell wall cross bridge (de Jonge *et al*, 1993; Stranden *et al*, 1997). A number of *Staphylococcus* species are known to be naturally resistant to lysostaphin because of presence of Fem like proteins which incorporate serine in place of a glycine in the pentaglycine cross bridge of the peptidoglycan (DeHart *et al*, 1995; Sugai *et al*, 1997; Ehlert *et al*, 2000). In a study involving FRET (fluorescence resonance energy transfer) assays, replacing 3^{rd} glycine of a GGGGG sequence with a serine was shown to abolish the activity of lysostaphin on the FRET substrate (Bardelang *et al*, 2009). Apart from *fem* genes, mutations in other genes have also been reported to confer resistance to lysostaphin. By screening a transposon mutant library of *S. aureus* for increased resistance to lysostaphin, Grundling *et al* (2006) found mutations in purine biosynthesis pathway and *lyrA* gene which codes for a uncharacterized membrane protein.

P128 is a chimeric protein consisting of catalytic CHAP domain of phage K ORF56 fused to the cell wall binding SH3b domain of lysostaphin (Paul *et al*, 2011). The CHAP domain of P128 (Lys16) by itself has bactericidal properties, which is accentuated by its fusion to SH3b domain (Paul *et al*, 2011). P128 is potent killer of *Staphylococcus* species but has no inhibitory activity on a number of other Gram positive and Gram negative bacteria tested (Vipra *et al*, 2012). Its

unique property to specifically kill *Staphylococcus* species including MRSA strains and lack of cytotoxicity on eukaryotic cells (George *et al*, 2012) suggests that P128 has the potential for treating a variety of infections caused by drug resistant strains of Staphylococci. P128 is currently undergoing safety and efficacy trials in Singapore for its use as a topical agent for decolonizing nasal carriage of *S. aureus* including MRSA (ClinicalTrials.gov Identifier: NCT01746654).

In this study we show that mutants of MRSA resistant to P128 have fitness defects and become sensitive to methicillin and other β-lactams. The mutations conferring resistance to P128 were mapped to *femA* gene linking the MOA of P128 to pentaglycine cross bridge in *S. aureus* peptidoglycan. In addition to GGGGG pentapeptide, P128 was shown to have cleaving activity on GGSGG and AGSGG, which are known to form cross bridges in *S. haemolyticus*, a coagulase negative *Staphylococcus* (CoNS) species causing nosocomial infections (Natoli *et al*, 2009)

Materials and methods

Plasmids, strains and culture conditions

The plasmids and strains used in this study are listed in Table 1. *S. aureus* cultures were routinely grown in Trypticase soy broth (TSB), LB broth or agar at 37^{o}C.

Generation of mutants resistant to P128

A diverse set of 50 *Staphylococcus* strains including clinical isolates of MRSA, human nasal commensals and strains isolated from canine pyoderma were selected for isolation of mutants resistant to P128. The cultures were grown in sub-MIC (0.5 X) concentrations of P128 and plated on MH agar containing 100 or 200 μg ml^{-1} of P128, incubated at 37^{o}C and observed for

the appearance of colonies up to 72 hours. Only 4 strains ((BK1, BK22, BK30 and CPD70) showed colonies growing in P128 containing plates, which were taken up for characterization. The lack of colonies in P128 plates inoculated with other strains was not investigated further.

Stability of mutant strains

The stability of P128 resistant mutant phenotypes was followed by repeated subcultures in LB broth without P128 up to 14 days. The cultures were tested for P128 resistance / sensitivity on day 5 and day 14 by lawn inhibition, CFU drop and MIC assays.

***In vitro* growth kinetics**

In vitro growth kinetics of P128 resistant mutants and the corresponding parent strains grown on LB agar and TS agar were performed in TSB or LB broth in triplicates. The cultures were suspended in TSB or LB broth to achieve an OD_{600} = 4.0. From this suspension 0.5 ml was inoculated into 50 ml of pre-warmed TSB or LB broth in a 250 ml conical flask. The cultures were incubated with shaking at 200 rpm at 37°C. The OD_{600} was determined at various time intervals during the incubation period. The experiment was performed in triplicate and repeated twice.

Spa typing

Spa typing of various *S. aureus* strains was performed according to the procedure described earlier (Shopsin *et al*, 1999). The amplified PCR products were visualized in ethidium bromide stained gel the DNA fragment was subjected to sequencing. The DNA sequence was compared with the sequences of known spa types using Ridom database (http://spa.ridom.de/spatypes.shtml.).

Binding of phage K to P128 mutants

Adsorption of Phage K to *S. aureus* RN4220, BK1, BK30, BK1R and BK30R was studied. The cultures were harvested from agar plates using LB broth and the OD_{600} was adjusted to 1.0.The cultures were infected with phage K at a MOI of 0.1, incubated at 37^{o}C for 20 minutes and centrifuged at 9200 g for 5 minutes. The supernatant was filtered through 0.2 µm filter and serially diluted supernatant fractions were spotted on *S. aureus* RN4220 lawn. The plates incubated overnight were observed for clearance of bacterial lawn in areas of spotting. *E .coli* C was taken as a negative control for phage K adsorption. The assay was performed in duplicate and repeated thrice.

In vivo phenotype in mice

Animal experiments were approved by the Institutional Animal Ethics Committee and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). GangaGen is registered with CPCSEA (registration No. 1193/c/08/CPCSEA dated 21/4/2008). In all the experiments, 8-9 weeks old 5 healthy female Balb/c mice were used in each group. For preparing inoculums, BK30 or BK30R cultures were grown overnight on nutrient agar containing 5% sheep blood. Colonies were harvested by flooding the plate with sterile 0.85% NaCl. The harvested cells were pelleted by centrifugation (5800 × g, 10 min) and resuspended in sterile 0.85% NaCl to obtain 1×10^7 - 4×10^7 CFU/µl. 200 µl of BK30 or BK30R was injected into mice intaperitoneally. Mice were observed for morbidity and mortality up to 48 hours. 3 animals were chosen randomly from each group and spleens and kidneys were collected for estimating the organ load.

Statistical analysis

The differences in CFU obtained in *in vitro* kill assays were analyzed by Students t-test. A value of $p < 0.05$ was considered significant. The statistical significance of CFU obtained from spleen and kidney of animals was evaluated by one-way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test. A value of $p < 0.01$ was considered to be significant. For comparison of mortality in animals infected with BK30 or BK30R, the Kaplan-Meier survival plot was drawn and the log rank test was performed using MedCalc (version12.1.4.0). A value of $p < 0.05$ was considered to be significant. Statistical analysis was done using Microsoft Excel and GraphPad InStat trial version from GraphPad Software.

Genomic DNA isolation

Genomic DNA of various *S. aureus* strains was isolated using Sigma bacterial genomic DNA extraction kit (Sigma Aldrich, USA) by a procedure suggested by the manufacturer with some modifications. Briefly, overnight cultures were centrifuged and the pellet was resuspended in 200 µl of Gram positive lysis solution provided in the kit. Glass beads (Sigma Aldrich) were added to the cell suspension and the contents were vortexed for 3 minutes. Next, lysostaphin (20 µg ml^{-1}) was added and the mixture was incubated at 37°C for 30 minutes. Lysis solution C was added followed by Proteinase K (40 µg ml^{-1}) and the tubes were incubated at 55°C for 10 minutes. DNA was precipitated by adding 200 µl of 100% ethanol and loaded onto a pre equilibrated column for further purification according to manufacturer's instructions.

GST Fusion constructs

For fusing the desired peptide sequence to C-terminus of GST and N-terminus of Staphylokinase (SAK), the peptide coding sequence was incorporated into the forward primer used for amplification of SAK coding region. The SAK coding region was purified from *S. aureus* (ATCC 8325) genomic DNA. The

sequences of primers used have been provided in Table S1. PCR conditions were as follows: denaturation at 95^{o}C for 5 min followed by 30 cycles of amplification at 95^{o}C, 50^{o}C and 72^{o}C for 30 s each and finally extension at 72^{o}C for 10 min. The PCR amplified pentaglycine-SAK fusion coding fragments were cloned into EcoRI site of pGEX 4T-1 to obtain pGDC279, pDGC281 and pGDC294. For generating GGSGG, AGSGG and GSSGG fusion, site directed mutagenesis was performed as described below. The sequences of the mutagenic primers have been given in Table S1.

Site-directed mutagenesis

Site Directed mutatagenesis was performed using a QuickChange Site-Directed Mutagenesis Kit (Stratagene). PCR conditions consisted of initial denaturation for 30 s at 95°C followed by 18 cycles of 95°C for 30 s, 55°for 1 min and 68°C for 6 minutes and final extension at 68°C for 5minutes. A 10 µl aliquot of the DpnI digested reaction mix was used to transform *E. coli* Top10 cells. The desired mutation in the clone was confirmed by DNA sequencing.

Protein purification

P128 and Lys16 were purified as described earlier (Paul *et al*, 2011). N-terminal His tagged lysostaphin was expressed in soluble form in *E. coli* ER2566 and purified through Ni^{2+}-NTA affinity matrix (QIAGEN) as described earlier (Saravanan *et al*, 2013). For GST fusion proteins, induced cell pellets were resuspended in 2 ml of 10 mM Tris buffer (pH 8.0) and were lysed by sonication. The lysed cells were centrifuged at 13,000 rpm for 10 min and the supernatant was taken for purification. The glutathione Sepharose 4 fastflow matrix column was equilibrated with 50 mM Tris–Cl buffer (pH 8.0) containing 1% Triton X-100 and 150 mM NaCl. After washing the bound GST fusion proteins were eluted using 10 mM reduced glutathione in 50 mM Tris–Cl, (pH 8.0) and dialyzed against 50 mM Tris–Cl buffer (pH 8.0). All the proteins used in the study were purified to approximately > 90% level. Protein estimation was performed by Bradford's dye binding method.

In vitro cleavage of GST fusion peptides

For monitoring cleavage of fusion peptides, 20 µg of purified GST fusion peptides were individually incubated with 10 µg of purified P128, Lys16 (Paul *et al*, 2011) or lysostaphin in the presence of 50 mM Tris–Cl (pH 8.0) at 37^{o}C for 16 hours. The samples were analyzed by 15% SDS-PAGE. The N-terminal sequence of the cleaved SAK fragment was determined by sequencing facility at Biochemistry department of Indian Institute of Science, Bangalore, India.

MIC, lawn inhibition spot assay and CFU drop assay

MIC was determined using a modified Clinical and Laboratory Standards Institute (CLSI) broth microdilution procedure described earlier (Vipra *et al*, 2012). For lawn inhibition spot assay, freshly grown culture was spread on a LB agar plate and was allowed to dry. Various amounts of protein were spotted on the dried plate and incubated overnight at 37^{o}C. Inhibition of bacterial growth resulted in a zone of clearance. CFU drop assay was performed as described earlier (Paul *et al*, 2011). Briefly, 2 µg of P128 was allowed to act on 10^{7} CFU of *S. aureus* in 200 µl saline for 1 hour at 37^{o}C with shaking at 200 rpm. Later, the reaction volume was filled up to 1 ml with LB and various dilutions were plated on LB agar plates to determine the residual bacterial counts. The CFU drop assays were done in duplicates and repeated three times.

Results

Characterization of P128 resistant mutants

Following the procedure described in materials and methods, colonies were observed in P128 containing plates inoculated with the three MRSA strains (BK1, BK22, BK30) and one *S. equorum* strain from canine pyoderma (CPD70) (Table 1). Based on this the frequency of resistance development was calculated to be approximately 3.5×10^{-6} to 2×10^{-7}. The induced

frequency of resistance against lysostaphin has been reported to range from very high to low (5.3 X 10^{-1} to 1.0 X 10^{-7}) (Climo *et al*, 2001). One putative resistant colony from each strain was taken up for further characterization. The colonies showed confluent growth when plated on 200 μg ml^{-1} P128 containing MH agar plates (Fig. 1a) and the MICs on the mutants was found to be > 2 mg ml^{-1} (Table 2). Four putative mutants designated as BK1R, BK22R, BK30R and CPD70R were checked for susceptibility to P128 by lawn inhibition assay and CFU reduction assay. By spotting 10 μg of P128 on parent *Staphylococcus* cultures clear zones of lysis were seen whereas there was no such clear zone in case of mutant cultures (Fig.1a). In CFU drop assay the parent strains and the sensitive control *S. carnosus* showed 3-6 log reduction in CFU upon treatment with P128 (10 μg ml^{-1}) for 1 hour. (Fig.1b). No significant reduction in CFU was obtained in any of the resistant mutants under similar conditions. This suggested that the mutants could resist the bactericidal action of P128. In order to make sure that the resistance to P128 is mediated through the catalytic domain of P128, susceptibilities of the mutants to Lys16 were assessed by lawn inhibition assay. As seen in Fig. 1c, 0.5 μg of P128 or 2.5 μg of Lys16 showed zones of clearance upon spotting on cultures of BK1 and BK30. However, even 20 μg of either of these proteins did not show any zones of clearance on BK1R and BK30R. Thus, it can be concluded that the BK1R and BK30R are resistant to the action of the catalytic domain of P128. In order to make sure that the resistant mutants were derived from their respective parents and to rule out any cross-contamination issues, the strains were subjected to spa typing by the procedure described in materials and methods. As seen in Table 2, the parent and the mutant strains belonged to the same spa type, which confirmed that they were isogenic.

Two unstable mutants reverted back to the parental phenotype

In order to determine the stability of the resistance phenotype the mutant strains were grown in the absence of P128 for 14 days with regular sub-culturing and were monitored for P128 sensitivity by MIC, lawn inhibition and CFU drop assay. It was observed that the mutant strains formed smaller colonies on LB agar plates compared to the parent strains (Fig. S1). Upon subculture, it was seen that BK1 and BK30 mutants were stable and had retained the small colony morphology and were resistant to P128 till 14 days (Fig. S1a). Interestingly CPD70R showed signs of reversion in the 2^{nd} passage and was fully reverted to P128 sensitivity after 5^{th} passage (Fig. S1a and Table S2). The reverted culture showed normal growth and had normal sized colonies on agar plates (Fig. S1). Similarly, BK22R also reverted to the parent phenotype of large colony and P128 sensitivity, though it took 9 sub-cultures to do so. The susceptibilities of the reverted BK22R and CPD70R cultures to P128 as measured by CFU reduction assays was similar to their parent strains (Fig. S1b and Table S2) confirming that they had lost the P128 resistance phenotype. This clearly demonstrated that some of the P128 resistant mutants were unstable and could revert back to the wild type phenotype upon subculture in the absence of P128.

P128 resistant mutants lose MRSA phenotype and are hypersensitive to β-lactams

Since P128 acts on cell wall, for understanding whether the resistance mutation brings any alterations in the susceptibility to cell wall inhibitors, MIC of oxacillin was tested against P128 resistant mutants. As seen in table 2, MIC of mutant derivatives of all the three MRSA strains BK1, BK22 and BK30, dropped drastically from > 256 μg ml^{-1} to < 0.1 μg ml^{-1}. The mutant strains were also found to be highly sensitive to methicllin as demonstrated by E-test in agar plates (Fig. S2) Thus, all the mutants were converted from MRSA to MSSA. MIC measurements of other β-lactams, viz., cephalothin, ceftizoxime, carbenicillin, ceftrixone, cefalexin,

amoxicillin, cefazolin, oxacillin showed that the mutant strains were sensitive to these compounds as well (data not shown). The sensitivity of the BK1R and BK30R mutant strains to a number of non- β-lactams antibiotics (kanamycin, streptomycin, clindamycin, vancomycin, mupirocin and moxifloxacin) remained unchanged (table S3), suggesting that the mutation had only affected the cell wall physiology of the bacteria in a specific way. Reduction in MIC of oxacillin and other β-lactams to sensitive levels indicated that the mutations in these strains might have altered proteins involved in peptidoglycan metabolism.

P128 resistant mutants show reduced rates of growth *in vitro*

Observations of colonies on LB and TS agar plates indicated that all the four mutant strains, BK1R, BK30R, BK22R and CPD70R formed smaller colonies probably because of their reduced growth rates. Growth kinetics of BK1, BK30 and their stable mutant derivatives in liquid cultures showed that BK1R and BK30R had significantly slower growth rates compared to the corresponding parent strains (Fig. S3). Additionally, the rate of growth of BK1R was much slower than BK30R in both LB and TS media. However, upon prolonged incubation up to 48 hrs, the colony size in plates and the OD_{600} values in liquid cultures of the mutants and the parental stains were similar.

Peptidoglycan from resistant cells is refractory to the action of P128

Since bacteriophage CHAP domain proteins possess muralytic activity and are able to cleave a variety of bonds in bacterial peptidoglycan (Pritchard *et al*, 2004; Becker *et al*, 2009; Fenton *et al*, 2010; Szweda *et al*, 2012; Gilmer *et al*, 2013;), resistance to P128 might involve changes in peptidoglycan. To find out whether the resistant mutants have alterations in peptidoglycan which will affect its cleavage by P128, enzymatic activity of P128 on peptidoglycan of wild type and

289 the corresponding mutants was studied by Zymogram using the protocol described earlier
290 (Lepeuple *et al*, 1998). Since the sensitivity of this assay is very high, the amount of proteins
291 used was quite low compared to the MIC values on the strains. As seen in Fig. 2, P128 was able
292 to cleave peptidoglycan of BK1 and BK30 parent strains very efficiently as a zone of clearing
293 could be seen with as little as 25 ng of P128. The peptidoglycans of the mutant strains (BK1R
294 and BK30R), however, were found be a poor substrates for P128 as a faint zone of clearing could
295 be seen only with concentrations higher than 250 ng of P128. Similarly, both BK1R and BK30R
296 were resistant to the action of Lys16 (Fig. 2), the enzymatic domain of P128. This demonstrated
297 that the mutations had caused alterations in the peptidoglycan of Staphylococci making them
298 resistant to the enzymatic action of P128.

299 **P128 mutants are resistant to phage K lysis though they can adsorb the phage**

300 Since P128 possesses the catalytic domain (Lys16) derived from *S. aureus* phage K, we tested
301 the susceptibility of P128 resistant mutants to phage K. As seen in Fig. 3a both BK1 and BK30
302 parent strains were sensitive to the action of phage K and abundant plaques were visible in 10^{-6}
303 diluted phage preparations. On the other hand, both BK1R and BK30R did not show plaque
304 formation under similar conditions. This clearly demonstrated that the P128 resistant strains had
305 become refractory to the action of phage K as well. We decided to find out whether the
306 resistance was because of inability of the phage to bind to the bacterial cells or it could bind but
307 could not replicate. As shown in Fig. 3b and C, *E. coli* C used as a negative control did not show
308 any significant binding to phage K as the phage titer seen on the indicator strain was same as that
309 of starting phage. A 4 log reduction in titer (from 1.3 $X10^7$ to 8.2 X 10^2 or 1.3 X 10^3 PFU ml^{-1})
310 of phage K upon binding to BK1 or BK30 indicated strong binding of the phage to these cells. A
311 very similar reduction in titer (from 1.3 $X10^7$ to 1.3 X 10^3 or 2.0 X 10^3 PFU ml^{-1}) obtained with

mutant strains showed that both BK1R and BK30R were able to bind to the phages almost as efficiently as the parent strains. This demonstrated that though phage K could bind to BK1R and BK30R, the cell lysis did not take place probably due to inability of phage K to cleave the altered peptidoglycan for injecting DNA to initiate replication.

P128 resistant mutants show reduced virulence

In order to assess any changes in the virulence of P128 resistant mutants, one of the stable mutants BK30R was compared with its parent strain for its ability to survive in mouse organs and to kill animals. Initial virulence studies conducted with BK30 parent strain showed that 10^9 CFU is required for killing > 50% of the animals. In two independent experiments BK30R was found to be attenuated in virulence as no mouse inoculated with this strain succumbed to infection (Fig. S4). On the other hand BK30 parent strain caused significant mortality (60%) in mice, which was statistically significant ($P < 0.05$). As shown in table S4 and a scatter plot in Fig S5, the total CFU obtained from the spleen of mice infected with BK30R was significantly less than what was found in spleen of mice infected with BK30. Similar CFU difference between BK30R and BK30 was also observed in kidneys. The CFU difference observed in spleen and kidney of mice infected with BK30 and BK30R was found to be statistically significant ($P < 0.01$). These experiments suggested that the mutation had affected the ability of bacteria to survive in animals and had reduced their ability to cause mortality. Thus there was a loss of fitness in P128 mutants in their ability to survive *in vitro* and *in vivo.*

Genetic characterization of P128 resistant mutants

The phenotypic characterization of P128 resistant mutants of *S. aureus* had shown loss of fitness *in vitro* and *in vivo* accompanied by conversion of MRSA phenotype to MSSA. As these mutants

also showed cross resistance to lysostaphin the mechanism of P128 resistance was expected to have a common element with lysostaphin resistance. Since, mechanism of lysostaphin resistance involves changes in *femA* gene (de Jonge *et al*, 1993; Kusuma *et al*, 2007), we decided to analyze *femA* genes of BK1R, BK22R and BK30R strains by PCR using primers shown in Fig S6a. The full length *femA* gene is reported to be 1302 bp coding for a protein of 433 amino acid residues. By PCR, all the parent strains (BK1, BK22 and BK30) which were MRSA and susceptible to P128 showed the presence of intact *femA* gene (Fig. S6b) with DNA sequence matching the reported sequence in the database. On the other hand, in BK1R (MSSA and resistant to P128), a premature stop codon was observed in the *femA* gene at 900^{th} bp position which resulted in termination of FemA polypeptide by 133 residues (Fig. S6b and c). Similarly in BK22R a stop codon at 459^{th} bp position truncated the polypeptide by 280 residues. In BK30R, PCR product of only ~350 bp was observed indicating that a part of the gene was deleted (Fig. S6b, lane 5). DNA sequencing showed a deletion of *femA* starting from 327^{th} bp and extending into initiation codon (ATG) of *femB* resulting in deletion of 325 amino acids in the FemA protein and abrogating formation of FemB. DNA sequence analysis of *femA* gene of P128 resistant mutants suggested that similar to lysostaphin, mutations in *femA* gene were causing resistance to P128. Interestingly, the reversion of P128 resistant BK22R to the P128 sensitive parent phenotype could be explained by conversion of premature stop codon TAA to CAA (coding for Gln) leading to restoration of expression of full length FemA protein. The *femA* mutations in BK1R, BK22R and BK30R could result in formation of monoglycine cross bridges instead of usual pentaglycine bridges seen in wild type strains of *S. aureus* (de Jonge *et al*, 1993; Stranden *et al*, 1997). These observations led us to investigate whether P128 mechanism of action could involve cleavage of pentaglycine cross bridge of *S. aureus*.

Cleavage of pentaglycine (GGGGG) and other sequence *in vitro*: Differences between substrate specificities of P128 and lysostaphin

Mapping of P128 resistance mutations in BK1R, BK22R and BK30R to *femA* genes suggested that target of P128 could be the pentaglycine cross bridge of *S. aureus* cell wall. In order to confirm this we investigated the cleavage of pentaglycine *in vitro* using GST fusion peptides (Fig. 4a). For this purpose a chimera of pentaglycine (GGGGG) and staphylokinase (SAK) was fused to C-terminus of GST (Fig 4b) in pGEX4T-1 vector as described in materials and methods. The 16 kDa SAK protein which is known to be expressed in soluble form in *E. coli* (Prasad *et al*, 2010), served the purpose of a reporter in this assay. Upon cleavage of pentaglycine linker the SAK fragment was expected to get separated from the GST fusion (Fig. 4b) and would thus be seen as a new protein band on a SDS-PAGE gel. As shown in Fig.4c, both lysostaphin and P128 were able to release SAK from the GST-GGGGG-SAK fusion protein with similar efficiency. In order to make sure that the cleavage is not because of the presence of lysostaphin SH3b domain in P128, we tested the cleavage of the fusion protein using Lys16, the catalytic domain of P128. It was seen that Lys16 alone could cleave the fusion protein on its own, thus ruling out the role of SH3b in pentaglycine cleavage. Additionally, SH3b domain alone did not show any activity on GST-GGGGG-SAK (Fig S7).This finding is consistent with bactericidal activity shown by Lys16 alone (Paul *et al*, 2011). N-terminal sequencing of the SAK fragment released upon cleavage showed that P128 was cleaving between 4^{th} and 5^{th} Gly residues of pentaglycine (Fig. S8). However, ability of P128 to cleave at multiple sites in the cross-bridge as reported for Lysostaphin (Warfield *et al*, 2013) cannot be ruled out at this stage.

Since there is no homology between lysostaphin and P128 CHAP domain, we surmised that, in spite of acting on the same substrate, there could be differences in their mechanism of action. In

order to see if the two proteins had differential preferences for substrates, we designed the following glycine containing polypeptides as GST fusions: GST-GAAAG-SAK, GST-GSSGG-SAK, GST-GGG-SAK, GST-GGSGG-SAK and GST-AGSGG-SAK. The lysostaphin producing strain of *Staphylococcus* (*S. simulans* biovar staphylolyticus), which is known to be resistant to the action of lysostaphin by virtue of presence of a Lysostaphin immunity factor (Lif) (Heath *et al*, 1989) carries GGSGG cross bridges in the peptidoglycan (Thumm & Götz, 1997) Similarly, *S. haemolyticus,* an emerging pathogen in nosocomial infections is known to carry GGSGG and AGSGG cross-bridges in its peptidoglycan (Billot-Klein *et al*, 1996).

It was seen that both P128 and lysostaphin did not cleave GST-GAAAG-SAK or GST-GSSGG-SAK fusion (Fig. 4d, right panel and 4e). Similarly, GST-GGG-SAK was found to be a poor substrate for both lysostaphin and P128, though P128 showed slightly better activity as seen by release of SAK fragment (Fig. 4d, left panel). Interestingly, GST-GGSGG-SAK on which lysostaphin did not show any activity could be cleaved by both P128 and Lys16 (Fig. 4c). The substrate specificities of P128 and Lys16 were again found to be same demonstrating that the cleavage specificity of P128 was because of its catalytic CHAP domain and SH3b did not contribute towards this specificity. Another sequence from *S. haemolyticus* peptidoglycan, AGSGG, was cleaved by P128 but not by lysostaphin (Fig 4e). These set of data proved that though both lysostaphin and P128 act on pentaglycine, there are significant differences in their substrate specificities.

In vivo **relevance of GGSGG and AGSGG cleavage: P128 can kill *S. haemolyticus***

In order to understand the physiological significance of differences in substrate specificities of P128 and lysostaphin we investigated the killing of Staphylococci harboring cross-brodge

sequences other than GGGGG. In a spot assay on *S. haemolyticus*, 2, 5 and 10 µg of P128 showed clear zones of lysis whereas lysostaphin failed to show any effect under these conditions (Fig 5a). In a cell killing assay using P128 or lysostaphin at 50 µg ml^{-1}, P128 reduced the CFU counts of *S. haemolyticus* by 6 logs, demonstrating a potent bactericidal activity on this species. No bactericidal effect was seen in lysostaphin treated cells (Fig 5b). This is consistent with the killing of *S. simulans* by P128 shown earlier (Saravanan *et al*, 2013). The potent activity of P128 on *S. haemolyticus* and *S. simulans* corroborates the biochemical finding of this study that, unlike lysostaphin, P128 is capable of cleaving a GGSGG and AGSGG containing cross bridge in *Staphylococcus* cell wall.

Discussion

Amongst the phage or bacteria derived murein hydrolases or Enzybiotics, mechanism of resistance has been studied in detail only in the case of lysostaphin (Climo *et al*, 2001; Kusuma *et al*, 2007). Attempts to isolate resistant mutants have been unsuccessful with other hydrolases including phage endolysins which probably means that any changes in their substrates are lethal to the cell (Loeffler *et al*, 2001; Schuch *et al*, 2002; Gilmer *et al*, 2013; Rodriguez-Rubio *et al*, 2013). In the current study, the frequency of induced resistance against P128 was found to be comparable to the lower range value (1.0×10^{-7}) reported for lysostaphin (Climo *et al*, 2001). The reason for unusually high frequency of lysostaphin resistant mutants (5.3×10^{-1}) observed in the earlier study is not known. The loss of susceptibility of P128 resistant mutants to Lys16, the catalytic domain of P128, proved that the mutants had become refractory to the enzymatic activity of P128. The slow growth rates of mutants resistant to P128 *in vitro* and reduced virulence in an animal model pointing towards loss of fitness *in vitro* and *in vivo*. The loss of fitness *in vitro* and or *in vivo* amongst antibiotic resistant bacteria has been reported earlier

(Andersson & Hughes, 2010). Instability of some of the P128 resistant mutants as indicated by reversion to the parent phenotype suggests that the resistance imposes burden on their fitness which drives the mutant bacteria towards loss of resistance phenotype. The loss of fitness shown by the stable P128 resistant mutants *in vitro* and *in vivo* suggest that these mutants are likely to be at a disadvantage for survival and spreading in a community. The reduction in virulence indicates that the mutant strains might be less pathogenic in humans as well. Similar loss of fitness shown by lysostaphin resistant mutants (Kusuma *et al*, 2007) indicates that P128 and lysostaphin might share a common mechanism of resistance. The ability of the mutant cells to bind to phage K shows that the phage receptors on the cell surface are intact. The resistance of these mutants to phage K lysis can possibly be attributed to the inability of phage K to cleave the peptidoglycan for injecting DNA into the cell. The reduction in cleavage efficiency of peptidoglycan purified from resistant mutants by P128, as shown by Zymogram analysis, suggested that increased resistance of the mutants to P128 resulted from changes in the substrate of P128. Thus presence of a common SH3b cell wall binding domain in lysostaphin and P128 cannot be the sole reason for cross resistance of P128 mutants to lysostaphin.

The prokaryotic CHAP domain proteins constitutes a family of murein hydrolases of bacterial and phage origin characterized by active site Cys and His residues (Zou & Hou, 2010). The MOA of CHAP domains at the level of cellular killing of bacteria is not understood since there are no reports on isolation of mutants of bacteria resistant to the action of CHAP domain proteins. Despite lack of any sequence or structural similarity of CHAP domains to lysostaphin (Rossi *et al*, 2009; Paul *et al* 2011), the P128 resistant *S. aureus* mutants were found to be resistant to lysostaphin. Earlier studies on lysostaphin resistant mutants involving changes in *femA* genes (Stranden *et al*, 1997; Kusuma *et al*, 2007) have proven that lack of FemA activity in

the cell can lead to reduction in growth rates and conversion of MRSA phenotype to MSSA. Thus it was not surprising that *femA* genes were found to be mutated in P128 resistant mutants. It is worth mentioning that in an earlier study, screening a library of transposon mutants had led to recognition of *femA* mutations being responsible for reduction of *S. aureus* virulence in a mouse model of infection (Mei *et al*, 1997). The reduced rates of growth in vitro, resistance to lysostaphin, conversion of MRSA to MSSA and attenuation of virulence in mice of P128 resistant mutants are consistent with the phenotypes of *femA* mutants reported earlier (Mei *et al*, 1997; Kusuma *et al*, 2007). The similarity of phenotypes shown by P128 resistant mutants and *femA* mutants suggests that changes in *femA* are contributing maximally towards P128 resistance. Since FemB protein is not expected to function in cells with inactive FemA (Ehlert *et al*, 1997), the mutant cells will have cross bridges containing only a single glycine. The mutations in *femA* also explain the β-lactam sensitivity of the mutants since it is known that *mecA* encoded PBP 2a protein, which is refractory to β-lactams, is unable to perform transpeptidation in cells lacking pentaglycine cross-bridges (Labschinski *et al* 1998; Rohrer and Berger-Bachi, 2003). In *femA* mutant *S. aureus* cells the transpeptidation reaction is carried out by the β-lactam sensitive PBP 2, thus rendering the cells susceptible to β-lactams. Further proof of the link of P128 MOA to the pentaglycine cross bridge came from the studies of *in vitro* digestion of fusion proteins containing GGGGG or its variants. The formation of monoglycine bridges in *femA* mutants (de Jonge *et al*, 1993; Stranden *et al*, 1997) and inability of P128 to cleave stretches with single glycine residues *in vitro* can explain the link between *femA* mutation and resistance to P128. In terms of the substrate specificities, we found that both P128 and lysostaphin act on pentaglycine substrate and also show feeble activity on triglycine substrate *in vitro*. However one important difference seen between lysostaphin and P128 is the ability of P128 to act on serine substituted

pentapeptide sequence (GGSGG, AGSGG) which is present in the cell walls of certain species of staphylococci such as *S. simulans* biovar s*taphylolyticus* and *S. haemolyticus* (Thumm & Götz, 1997; Billot-Klein *et al*, 1996). Further, the *in vivo* relevance of the ability of P128 to cleave serine containing GGSGG or AGSGG sequence has been established by demonstration of its bactericidal activity on *S. simulans* biovar *staphylolyticus* (Saravanan *et al*, 2013) and *S. haemolyticus*. Because of this property the spectrum of activity of P128 on *Staphylococcus* species is likely to be broader than that of lysostaphin.

The loss of fitness and conversion of the mutants to β-lactam sensitivity and its ability to inhibit certain lysostaphin resistant variants of *Staphylococcus* make P128 an attractive candidate for clinical development.

Acknowledgements: We thank J Ramachandran and M Jayasheela for their support, encouragement and inputs and S. Anand Kumar for giving suggestions for improving the manuscript. We would like to thank the anonymous reviewers for providing critical inputs. We also thank the DSP team of GangaGen for providing purified proteins. We are grateful to Barry Kreiswirth, PHRI, New York, USA, for providing us the MRSA isolates.

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Table 1. Strains and plasmids used in this study

Strains / plasmids	Relevant characteristics	Source / Ref
S. aureus		
BK1, BK22 and BK30	Clinical isolates of MRSA	Barry Kreiswirth, Public Health Research Institute, New Jersey, USA
BK1R, BK22R and BK30R	P128 resistant mutants	This study
S. equorum CPD70	Canine pyoderma isolate	Veterinary college hospital and Lakeside veterinary clinic, Bangalore, India
S. carnosus (no ATCC51365)	Antibiotic sensitive strain	ATCC
S. aureus RN4220	Propagating host for phage K	Dr. Richard Novick (Skirball Institute, New York, NY, USA).
S. haemolyticus SM131		DSMZ DSM20263
Phage K (NC07814-02)		NCTC
Plasmids		
pGEX-4T-1	GST fusion vector	GE Healthcare
pGDC279	GST-GGGGG-SAK fusion in pGEX-4T	This study
pGDC281	GST-GAAAG-SAK fusion in pGEX-4T	This study
pGDC294	GST-GGG-SAK fusion in pGEX-4T	This study

pGDC293	GST-GGSGG-SAK fusion in pGEX-4T	This study
pGDC397	GST-AGSGG-SAK fusion in pGEX-4T	This study
pGDC399	GST-GSSGG-SAK fusion in pGEX-4T	This study

Table 2. MICs of P128 and Oxacillin against sensitive and P128 resistant mutants.

S. aureus* strains	**Spa type**	**MIC (µg ml^{-1})**	
		P128	**Oxacillin**
BK1	t002	1-2	>256
BK1R	t002	>2000	0.047
BK22	t018	4-8	>512
BK22R	t018	>2000	0.064
BK30	t004	2-4	>256
BK30R	t004	>2000	0.032
***S. equorum* strains**			
CPD70	ND	16-64	1
CPD70R	ND	2000	0.5

*BK1, BK22 and BK30 are MRSA strains while CPD70 is a methicillin sensitive *S. equorum.*

673 **Figure legends**

674 **Fig 1**. Susceptibility of *S. aureus* parent and mutant derivatives to P128. **(a)** Comparison of P128
675 sensitivity of the parent and the resistant variants analyzed by either incorporating P128 in agar
676 media or by spotting P128 on plates inoculated with the parent or the resistant variant. In the
677 figure CPD70 has been shown as an example; similar results were obtained with other parent and
678 the mutant derivatives. **(b).** Susceptibilities of the four parent and P128 resistant variants as
679 assessed by CFU reduction assay. *S. carnosus* was used as a P128 sensitive control strain. The
680 assay was performed in duplicate and was repeated thrice. The values represent mean of CFU
681 obtained in three experiments and the error bars represent standard deviation. The difference in
682 CFU observed between the parent and the mutant strains was found to be statistically significant
683 ($p < 0.001$). **(c).** Susceptibilities of BK1, BK1R, BK30 and BK30R to P128 and Lys16 by lawn
684 inhibition assay using 0.5-20 µg of protein. The assay was performed thrice with similar results.

685 **Fig 2.** Zymogram analysis of BK1 and BK30 parent and resistant variants using P128 and Lys16.
686 Cell walls prepared from the respective strains were run on a SDS-PAGE and processed as
687 described in materials and methods. 25-500 ng of P128 or 0.60-20 µg of Lys16 was used for
688 assessing the enzymatic activity on peptidoglycans of sensitive or resistant variants. All the
689 zymograms were repeated at least three times. The results of one of the experiments have been
690 shown here.

691 **Fig 3.** Binding of phage K to BK1 and BK30 parent and resistant variants. **(a)** Plaque formation
692 using 10^{-6} diluted phage K on BK1, BK1R, BK30 and BK30R. **(b)** Titration of residual phage K
693 after binding to various strains. Phage K was allowed to bind to various cultures and increasing
694 dilutions of unbound phage in the supernatant were spotted on a lawn of phage K sensitive *S.*
695 *aureus* RN4220. Absence of lysis or plaques at higher dilutions indicates binding of phage K to a

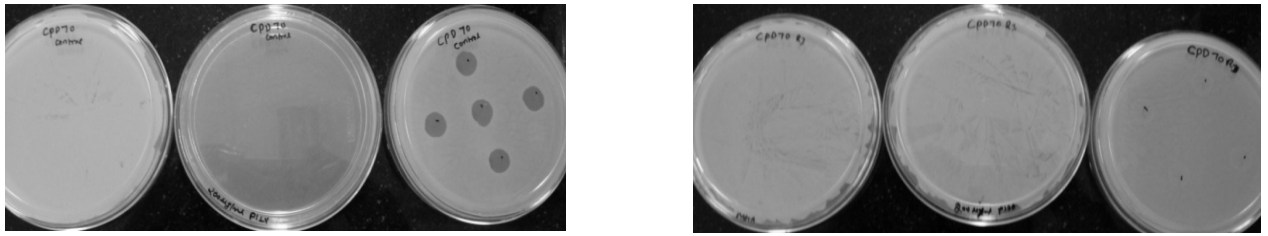
particular culture. Phage dilutions are indicated at the top of the panel, N indicates neat undiluted supernatant. **(c).** Residual phage PFU obtained after binding to various strains. The number indicate mean residual PFU observed in three independent binding experiments performed in duplicate with error bars indicating standard deviation. The difference in PFU seen between the parent and the mutant derivatives of BK1 and BK30 strains was not statistically significant ($P > 0.05$).

Fig 4. *In vitro* cleavage of GST-GGGGG-SAK, GST-GGSGG-SAK, GST-GGG-SAK, GST-AGSGG-SAK, GST-GSSGG-SAK, or GST-GAAAG-SAK by P128, Lys16 or lysostaphin. **(a)** Diagramatic representation of Lys16, P128 and lysostaphin showing catalytic CHAP domain and SH3b binding domains. **(b)** A linear diagram of GST:SAK showing position and sizes of the two domains of the fusion protein linked by GGGGG, GGSGG, GGG, AGSGG, GSSGG or GAAAG. **(c).** Cleavage of the fusion proteins by P128, lysostaphin or Lys16 analyzed by SDS-PAGE. The cleavage reaction was performed by the procedure described in materials and methods and the reaction products were loaded on a SDS-PAGE. P128, lysostaphin and Lys16 incubated in the absence of GST fusion proteins are shown on the left side of the panel. The middle and right side of the panel show the reaction products obtained after incubating GST-GGGG-SAK or GST-GGSGG-SAK fusion proteins with P128, Lysostaphin or Lys16 proteins. **(d)** *In vitro* cleavage of GGG (left panel) and GAAAG (right panel) fusion peptides. **(e)** *In vitro* cleavage of AGSGG and GSSGG fusion peptides by P128 and lysostaphin. Lys16 is the catalytic domain of P128. The assay was repeated thrice and results from one of the experiments are shown here. Lyso and MW stand for lysostaphin and molecular size marker respectively.

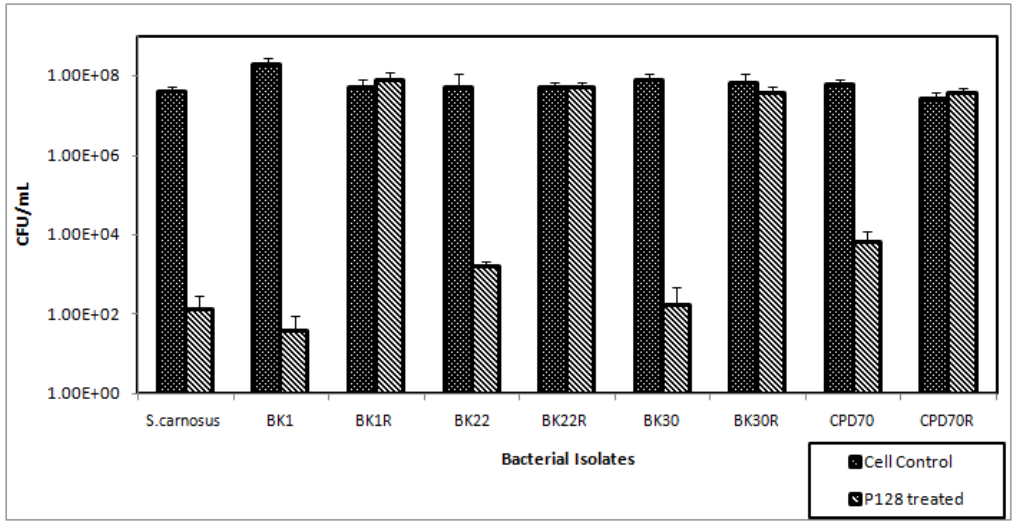
Fig 5. Inhibition of *S. haemolyticus* by P128. **(a).** Activity of P128 and lysostaphin on *S. carnosus* and *S. haemolyticus* by spot assay in LB agar plates. **(b)** *In vitro* bactericidal activity of

P128 and lysostaphin (50 µg/ml) on *S. haemolyticus* . The numbers represent mean of CFU values obtained in triplicates, +/- SD. Both the experiments were repeated thrice and results from one of the experiments are shown here.

(a)


Parent
P128 resistant
Cell control
P128 in medium
P128 spotted
Cell control
P128 in medium
P128 spotted

(b)


1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
CFU/mL
S.carnosus
BK1
BK1R
BK22
BK22R
BK30
BK30R
CPD70
CPD70R
Bacterial Isolates
Cell Control
P128 treated

(c)

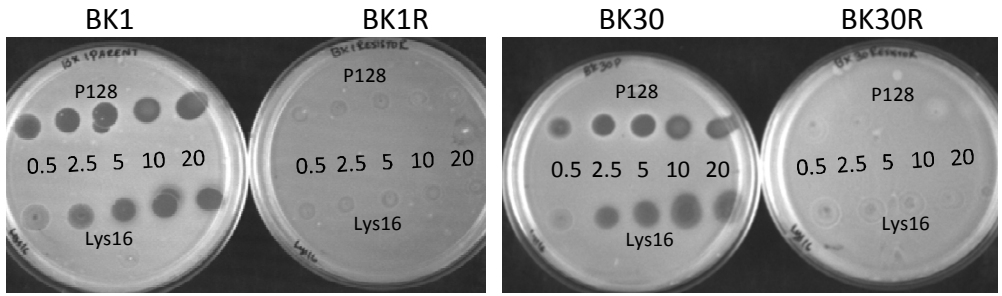

BK1
BK1R
BK30
BK30R
P128
0.5 2.5 5 10 20
Lys16

Fig. 1

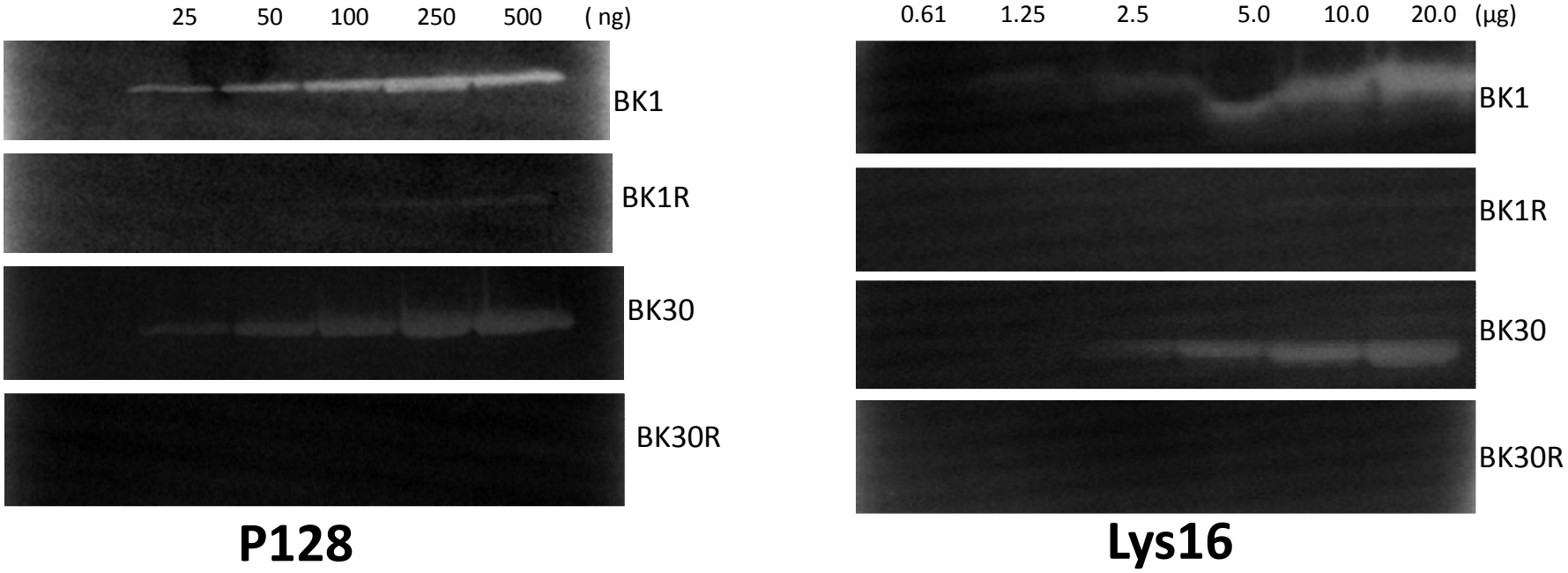
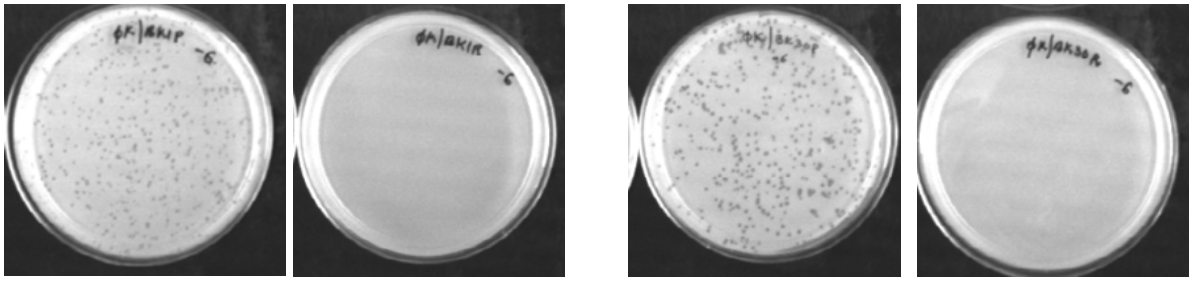

25
50
100
250
500
(ng)
BK1
BK1R
BK30
BK30R
P128
0.61
1.25
2.5
5.0
10.0
20.0
(µg)
BK1
BK1R
BK30
BK30R
Lys16

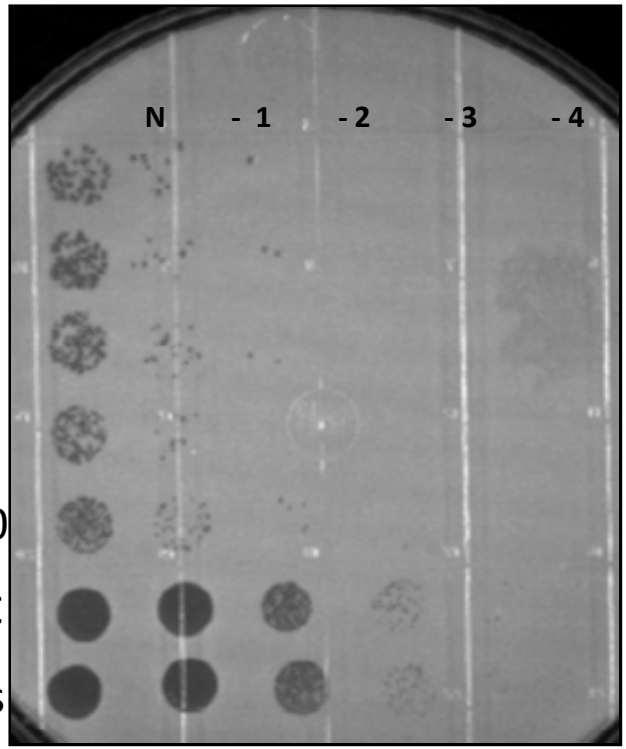
Fig. 2

(a)



BK1 BK1R BK30 BK30R

(b)



(c)

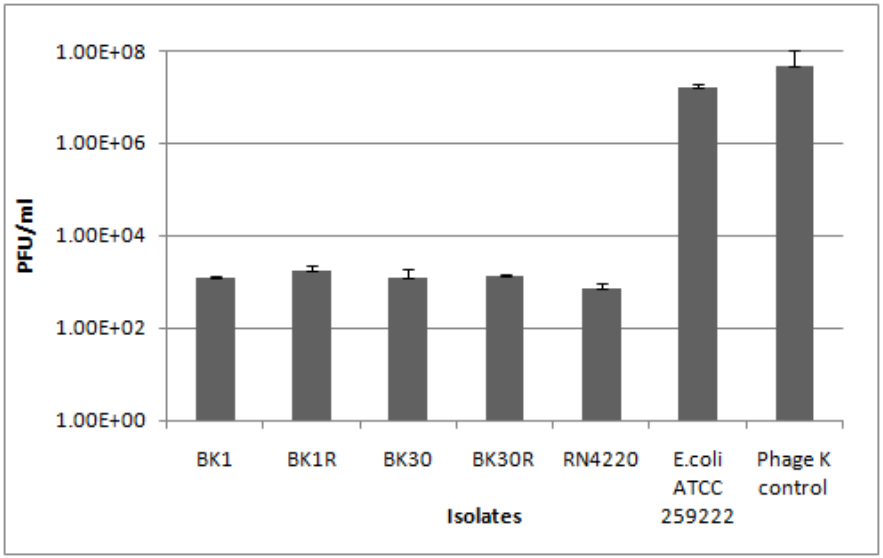


Fig. 3

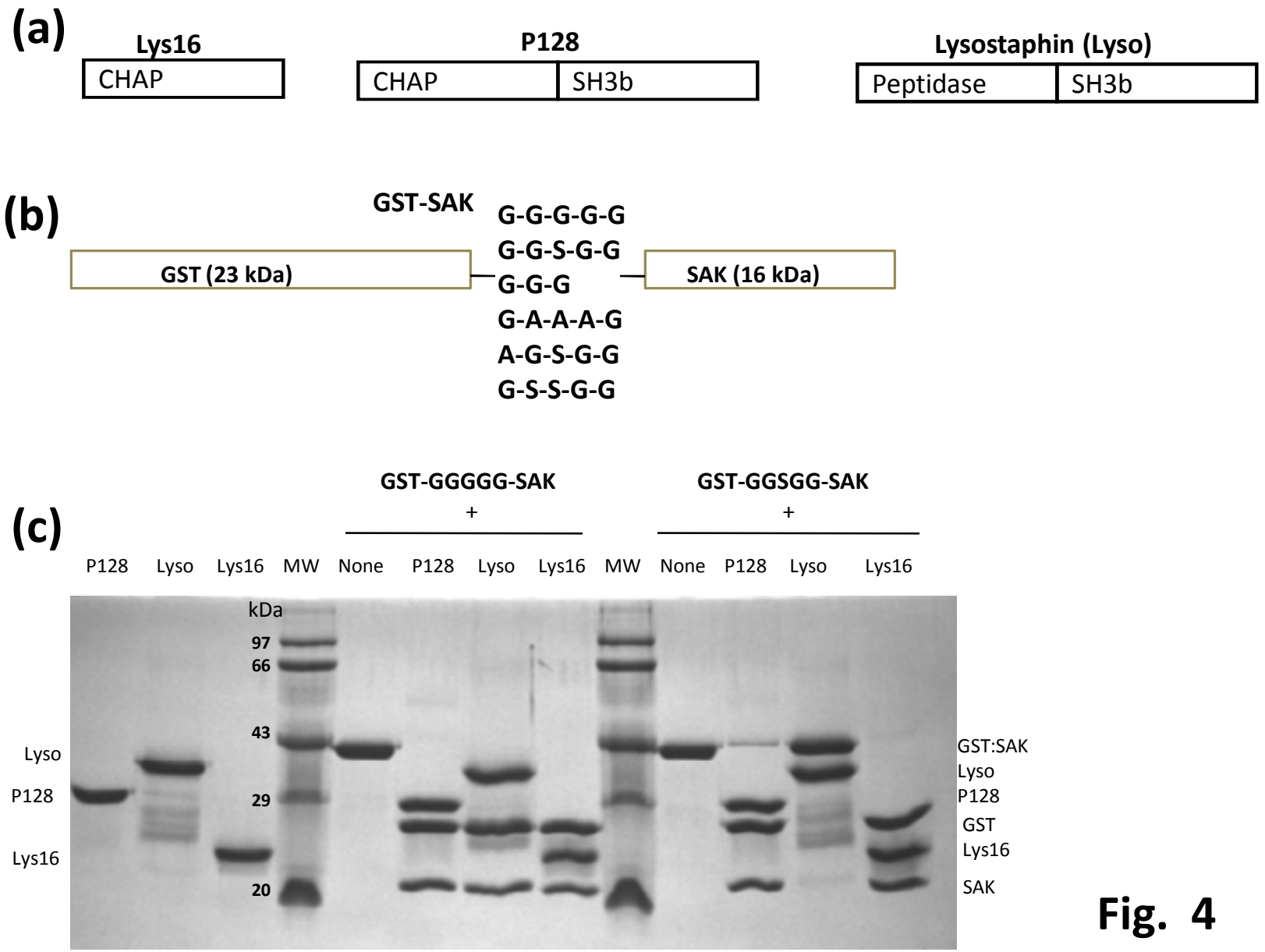

(a)
Lys16
CHAP
P128
CHAP
SH3b
Lysostaphin (Lyso)
Peptidase
SH3b
(b)
GST-SAK
G-G-G-G-G
G-G-S-G-G
G-G-G
G-A-A-A-G
A-G-S-G-G
G-S-S-G-G
GST (23 kDa)
SAK (16 kDa)
(c)
GST-GGGGG-SAK
+
GST-GGSGG-SAK
+
P128
Lyso
Lys16
MW
None
P128
Lyso
Lys16
MW
None
P128
Lyso
Lys16
kDa
97
66
43
29
20
Lyso
P128
Lys16
GST:SAK
Lyso
P128
GST
Lys16
SAK

Fig. 4

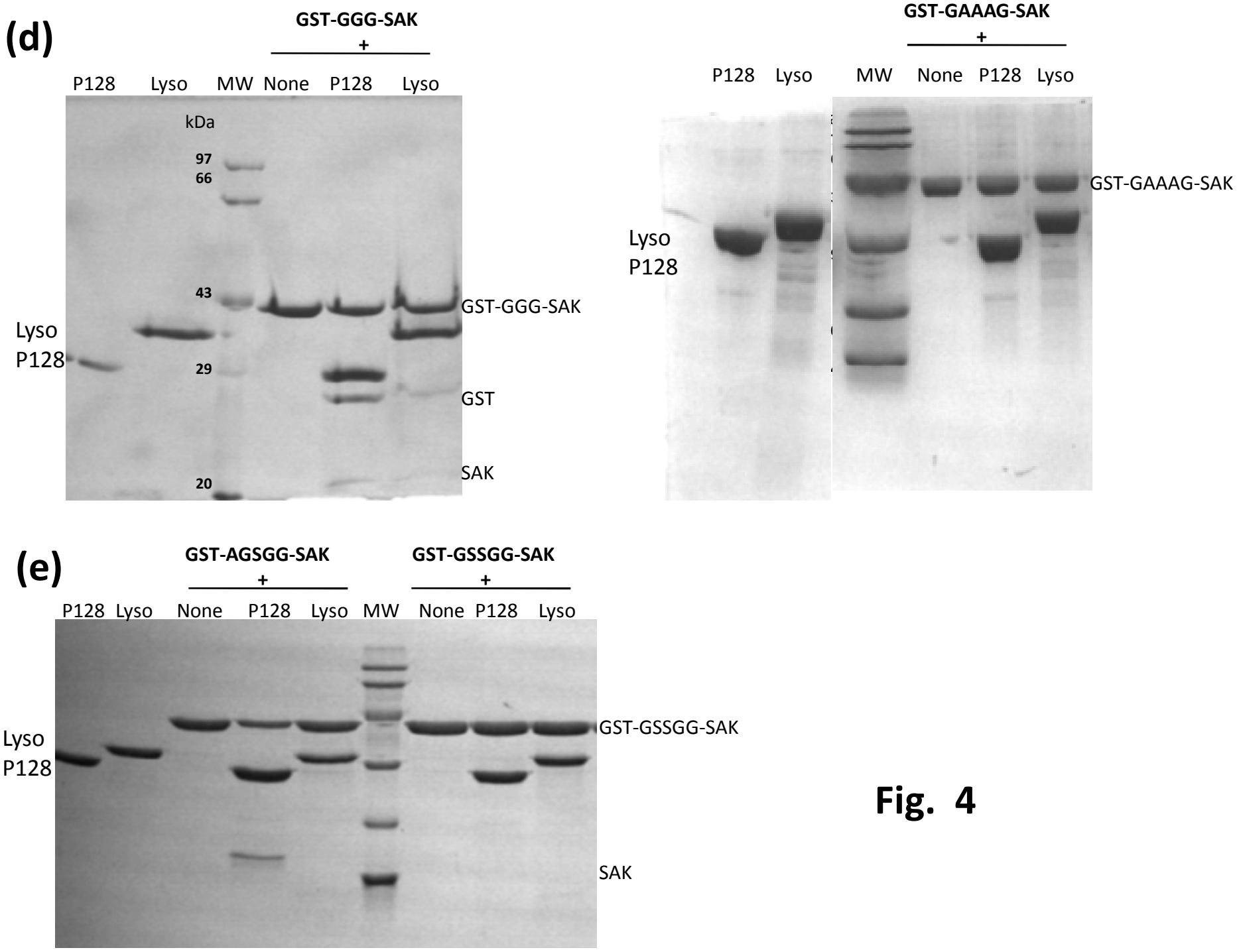
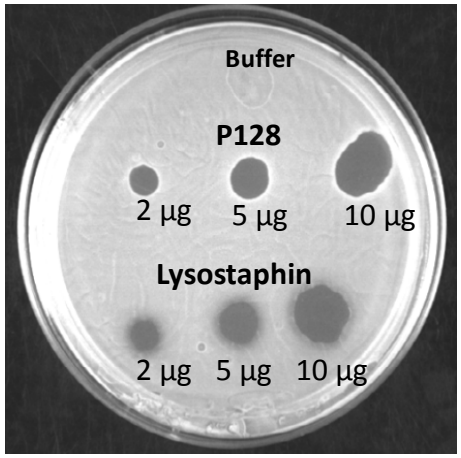
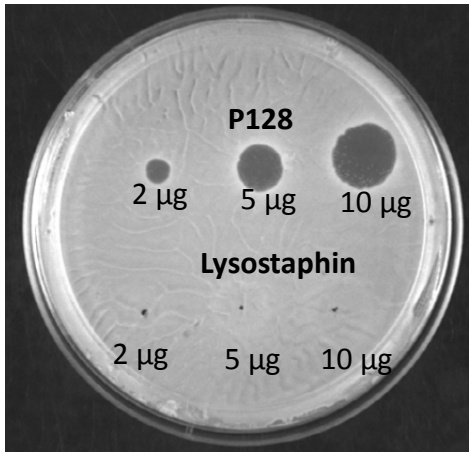

(d)
GST-GGG-SAK
+
P128
Lyso
MW
None
P128
Lyso
kDa
97
66
43
29
20
Lyso
P128
GST-GGG-SAK
GST
SAK
GST-GAAAG-SAK
+
P128
Lyso
MW
None
P128
Lyso
Lyso
P128
GST-GAAAG-SAK
(e)
GST-AGSGG-SAK
+
GST-GSSGG-SAK
+
P128
Lyso
None
P128
Lyso
MW
None
P128
Lyso
Lyso
P128
GST-GSSGG-SAK
SAK

Fig. 4

(a)



S. carnosus



S. haemolyticus

(b)

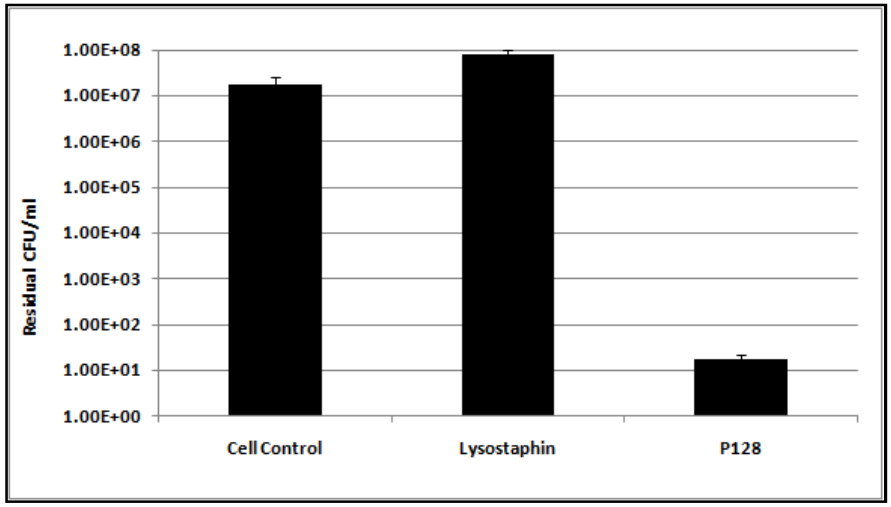


Fig. 5