# Processing and functional display of the 86 kDa heterodimeric penicillin G acylase on the surface of phage fd

Raymond M. D. VERHAERT*†[1], Jan VAN DUIN† and Wim J. QUAX†

*Pharmaceutical Biology, University Centre for Pharmacy, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands, and †Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands

The large heterodimeric penicillin G acylase from *Alcaligenes faecalis* was displayed on the surface of phage fd. We fused the coding sequence ($\alpha$ subunit–internal peptide–$\beta$ subunit) to the gene of a phage coat protein. A modified g3p signal sequence was used to direct the polypeptide to the periplasm. Here we show that a heterodimeric enzyme can be expressed as a fusion protein that matures to an active biocatalyst connected to the coat protein of phage fd, resulting in a phage to which the $\beta$-subunit is covalently linked and the $\alpha$-subunit is non-covalently attached. The enzyme can be displayed either fused to the minor coat protein g3p or fused to the major coat protein g8p. In both cases the penicillin G acylase on the phage has the same Michaelis constant as its freely soluble counterpart, indicating a proper folding and catalytic activity of the displayed enzyme. The display of the heterodimer on phage not only allows its further use in protein engineering but also offers the possibility of applying this technology for the excretion of the enzyme into the extracellular medium, facilitating purification of the protein. With the example of penicillin acylase the upper limit for a protein to become functionally displayed by phage fd has been further explored. Polyvalent display was not observed despite the use of genetic constructs designed for this aim. These results are discussed in relation to the pore size being formed by the g4p multimer.

Key words: *Alcaligenes faecalis*, penicillin amidase, phage display, phage-enzyme, protein secretion.

## INTRODUCTION

In 1985, Smith [1] showed that small peptides can be expressed on the surface of the filamentous phage fd. A small peptide was expressed as a fusion to the N-terminus of g3p coat protein resulting in the 'display' of that peptide. It was shown that it is possible to select for a phage encoding a specific peptide by affinity binding. This principle has been exploited to isolate specific peptides from large libraries of phages, which differ in the peptide that they display on their surface. The selection of a desired peptide, e.g. by binding it to an antibody, automatically leads to the co-selection and isolation of the DNA that codes for the peptide. It has been reported that a single phage particle can be selected out of a population of $10^7$ by multiple rounds of selection and enrichment (reviewed in [2]).

Initially the application of this technique was restricted to small peptides. Later it was found that protein domains [3] and even complete proteins can be functionally expressed as fusion proteins to g3p. The expression of Fab fragments suggests the possibility of dimer formation during phage assembly [4]. It was demonstrated that an enzyme, alkaline phosphatase, can be expressed in an active state on the surface [5]. In addition, $\beta$-lactamase was displayed on the surface of phage fd as a fusion with g3p coat protein. A suicide inhibitor was used to selectively enrich a mutant enzyme [6]. A glutathione transferase with a novel binding specificity has been selected by using phage display; unfortunately the activity of this protein was severely decreased [7].

The functional display of monomeric enzymes has opened the way to using phage fd as a vehicle to select for enzymes with altered properties. To explore the limits of phage display for enzyme selection we wished to investigate whether it is possible to display a heterodimeric enzyme. Penicillin G acylase (penicillin amidase or penicillin amidohydrolase, EC 3.5.1.11) catalyses the hydrolysis of penicillin into phenylacetic acid and 6-amino-penicillanic acid. The enzyme is translated from a single gene into one polypeptide [8]. The enzyme is preceded by a signal sequence that directs the polypeptide to the periplasm. There it is matured into two dissimilar subunits. For *Alcaligenes faecalis* penicillin acylase the size of the $\alpha$ subunit is 23 kDa and that of the $\beta$-subunit is 63 kDa [9]. Concomitantly with the generation of the $\alpha$ and $\beta$ subunit, an internal peptide spacing these subunits is removed. This maturation into the active $\alpha$–$\beta$ form is essential for activity [10,11] and it requires the presence of specific amino acid residues both in the internal peptide and in the $\beta$-subunit [12]. The peptide removal is thought to occur via an autocatalytic mechanism [13]. Thus the expression of penicillin acylase on phage requires not only the fusion and folding of a large polypeptide but also post-translational proteolysis to form a heterodimer.

There is great interest in penicillin G acylases with novel specificities for use in the enzymic synthesis of new $\beta$-lactam derivatives. Random mutagenesis combined with growth on specific substrates has been used to obtain penicillin acylases with new side-chain specificities [14,15]. However, the range of substrates useful for selective growth is limited. The phage display technique, in combination with the technique of random mutagenesis, offers a new method for directed evolution towards an altered substrate specificity. For such an application the expression and analysis of the enzyme on phage are essential. The functional presence of penicillin G acylase on the surface of a phage is not obvious, because the large protein also needs to be processed and transported through the cell membrane. To facilitate the detection and analysis of the enzyme on phage a

Abbreviations used: IPTG, isopropyl $\beta$-D-thiogalactoside; NIPAB, 6-nitro-3-phenylacetamido)benzoic acid; PGA, penicillin G acylase.

[1] To whom correspondence should be addressed in Groningen (e-mail r.m.d.verhaert@farm.rug.nl).

polyvalent display would be desirable. Here we present the functional expression of the 86 kDa heterodimer on phage fd and show that both g3p and g8p can be used to anchor the enzyme to the phage. Different genetic constructs were explored to increase the display level of the enzyme. The results are explained in terms of physical size limitations of the outer membrane pore in relation to the enzyme-displaying phage.

## MATERIALS AND METHODS

### Materials

#### Strains

*Escherichia coli* TG1 was Sup E, K 12 Δ(lac-pro), thi, hsd D5/F', traD 36, pro AB, lacIq, lac Z Δ-M15; *E. coli* HB 2151 was K12 Δ(lac-pro), ara, nal$^r$, thi/F', pro AB, lacIq, lac Z-ΔM15.

#### Oligonucleotides

The following primers were used to construct the plasmids shown in Figure 1. Italics denote the newly created restriction sites. PGA-F2, 5′-GT TTG ATC TTG GGT TGG GC*G GCC CAG CCG G*CC ATG GCG CAA GTG CAG TCG G-3′ (*Sfi*I); PG-R1, 5′-CAG GTG CCG CAC T*GC GGC CGC* AGG CTG AGG CTG AAT CAA C-3′ (*Not*I); Bio-450, 5′-*G GCC G*GT CCA GGA *GGT CC*T CAC CAT CAC CAT CAC CA*T*-3′ (*Eag*I, *Eco*O109, *Bsp*EI); Bio-452, 5′-*C CGG A*TG GTG ATG GTG ATG GTG A*GG ACC* TCC TGG AC*C*-3′ (*Bsp*EI, *Eco*O109, *Eag*I); Bio-543 (8F), AGT TTC AT*T CCG GAT CC*C TAG GGC G*CT GCA G*AG GGT GAC GAT C-3′ (*Bsp*EI, *Bam*HI, *Pst*I); Bio-458(8R), 5′-GA GCC TTT AAT *GAA TTC* TTA TTA TCA GCT TGC-3′ (*Eco*RI); Bio-560, 5′-ATT TA*G GAT CCC GGG* GGC G*CT GCA G*GG ACT GTT GAA AGT TG-3′ (*Bam*HI, *Sma*I, *Pst*I); Bio-561, 5′-C GAA T*AG ATC T*TC ATT AAA GCC AG-3′ (*Bgl*II).

#### Other materials

6-Nitro-3-(phenylacetamido)benzoic acid (NIPAB), Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate were purchased from Sigma (St. Louis, MO, U.S.A.). Isopropyl β-D-thiogalactoside (IPTG) was from Promega.

### Construction of the penicillin G acylase phagemids

The gene from *A. faecalis* was isolated from the plasmid pAF1 [9] with the primers PGA-F2 and PGA-R1. PCR was performed with *Pfu* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.) with the use of a protocol in which the annealing and extension temperatures were low: 4 min at 94 °C followed by 10 cycles of 30 s at 94 °C, 1 min at 37 °C and 10 min at 55 °C, followed by 30 cycles in which the extension time was, at first, 8 min, but increased by 15 s in each cycle (for the first of these cycles, conditions used were 30 s at 94 °C, 1 min at 55 °C, and then 8 min at 72 °C). At the end, DNA production was finished with 10 min at 72 °C.

The modified gene fragment was placed behind the signal peptide of the g3p signal sequence in pCANTAB 5E (Pharmacia, Uppsala, Sweden), thereby removing the signal sequence of the penicillin G acylase (pPGA-E-g3p). Different plasmids were then constructed with modifications in the linker between penicillin G acylase and the coat protein. Plasmid pPGA-H-g3p, containing a hexahistidine tag between the enzyme and the coat protein, was constructed from this first plasmid by replacing part of the E-tag for the oligonucleotides Bio-450 and Bio-452 (Figure 1).

The plasmid pPGA-Amb-g8p formed the basis of the four constructs that were used to compare the display efficiency (see below). It was constructed as follows: the g8p gene without a signal sequence was isolated by PCR (Bio458/543) from M13KO7 (Pharmacia) with concomitant incorporation of a *Bam*HI site close to the amber site. Insertion of the g8p gene (*Eco*RI/*Bsp*EI digestion) into pPGA-H-g3p, opened with the same enzymes, also removed the residual part of the E-tag.

The plasmid pPGA-g3p was constructed by inserting the *Bam*HI/*Bgl*II-digested Bio560/561 PCR-amplified pPGA-H-g3p back into the same vector opened with *Bam*HI, resulting in a phagemid with only one *Bam*HI site and its nonsense (TAG) codon replaced by a glycine residue.

The plasmids pPGA-Amb-g8p and pPGA-g3p were digested with *Pst*I and *Eco*RI. The *Pst*I–*Eco*RI fragment from pPGA-Amb-g8p containing the g8p coding sequence was inserted into the *Eco*RI–*Pst*I vector fragment of the pPGA-g3p plasmid (lacking the g3p coding sequence), yielding pPGA-g8p. Similarly, pPGA-Amb-g3p was created by insertion of the g3p coding sequence into the *Eco*RI–*Pst*I vector fragment pPGA-Amb. The sequence of the constructs (Figure 1) was verified by PCR, restriction analysis and sequencing.

### Transformation and phage rescue

*E. coli* TG1 cells were transformed by using the temperature shock method in transformation buffer [5 % (v/v) DMSO/10 % (w/v) poly(ethylene glycol)/50 mM $MgCl_2$ in Luria–Bertani medium]. Typically, 10 ng of vector and 10 ng of fragment were used. Phages were rescued by the addition of $10^{10}$ plaque-forming units/ml (i.e. a phage-to-bacterium ratio of 30:1) M13KO7 helper phage particles to exponential-phase transformants followed by 16 h of growth on selective kanamycin/ampicillin glucose-depleted Luria–Bertani medium. Typically, $10^8$–$10^9$ infective phages were obtained per ml of the original culture. Routinely, phages were precipitated with 4 % (w/v) poly(ethylene glycol) (average molecular mass 4 kDa)/0.6M NaCl, resuspended to $10^{11}$–$10^{12}$ colony-forming units/ml and passed through a 0.45 μm pore-size filter (Millipore, Bedford, MA, U.S.A.). Precipitation was performed both for concentrating the phages and for removing contaminating soluble protein.

### Titre determination

The number of phage particles in suspension was determined in two ways. First, the number of infective phage particles was counted by mixing 200 μl of a $10^2$–$10^8$-fold diluted phage suspension and 200 μl of exponential-phase *E. coli* TG1 cells and plating them immediately. Secondly, a determination was made from the total amount of protein in the phage suspension. Each phage particle contains a fixed amount of g3p, g6p, g7p and g9p (i.e. $5.4 \times 10^{-16}$ mg per phage particle [16,17]) but the amount of g8p depends on the size of the DNA that is packed. Therefore a pPGA-g3p phage particle (6860 nt) weighs approx. $3.0 \times 10^{-14}$ mg and a pPGA-g8p particle $2.6 \times 10^{-14}$ mg.

### Analysis of the constructs

Non-suppressor cells (*E. coli* HB2151) were infected with the rescued phages and selected for antibiotic resistance. IPTG was used for induction of the production of penicillin G acylase. Cell fractions were obtained as follows. Cells were collected by centrifugation (2000 ***g***) and resuspended in 1/100 vol. of PBS. Cell-free extracts were obtained by sonication (30 s, 10 pulses,

9 W) (Sonics & Materials). Cell debris was removed by centrifugation (10 min at 13000 ***g***). Protein was extracted from the periplasm by the addition of EDTA (1 mM) to the PBS. After a 10 min incubation on ice, cells were removed by centrifugation (10 min at 13000 ***g***). Protein concentration was determined by the Bradford method (Pierce).

### Phage electrophoresis and immunostaining

Phage particles (M13KO7, pPGA-Amb-g8p and pPGA-Amb-g3p) ($10^{10}$–$10^{11}$) were loaded on a 2% (w/v) agarose gel in 25 mM Tris/250 mM glycine buffer (pH 8.6) and run at 10 V for 16 h. For detection of DNA, part of the gel was stained by ethidium bromide in 0.1 M NaOH for 1 h followed by destaining overnight in water. For immunostaining, phages were electroblotted to nitrocellulose (16 h, 30 V). The blots were blocked by 2% (w/v) ovalbumin in PBS containing 0.1% (v/v) Tween 20. After the blots had been rinsed, rabbit antiserum specific for penicillin G acylase was added to the blot in the same medium in the presence of a 100-fold excess of bovine serum (16 h, 4 °C). The blot was incubated with phosphatase-labelled goat anti-rabbit IgG and developed with Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate.

Modification of the g3p was studied with mouse monoclonal antibodies (PSKAN3; MoBiTec, Göttingen, Germany). The blot was incubated with the monoclonal antibody in PBS (80 ng/ml) for 10 h. Detection of the antibody was performed with phosphatase-conjugated goat antibodies against mice.

### Chelation

Immobilization on $Ni^{2+}$-nitrilotriacetate agarose (Qiagen) was performed as follows. Phage suspension (0.4–1.0 ml) or cell-free extract (100 $\mu$l) was incubated with 40 $\mu$l of a 50% (w/v) column material slurry in buffer [50 mM sodium phosphate (pH 8.0)/300 mM NaCl] for 1 h at 0 °C. The slurry was rinsed three times with 1 ml of the same buffer. Elution was with 100 $\mu$l of 0.5 M imidazole in buffer.

### Enzyme purification and enzyme kinetics

The enzyme was purified as described elsewhere [9]. Activity was determined spectrophotometrically by the hydrolysis of 1 mM of NIPAB in 50 mM phosphate buffer, pH 7.5 [18]. The reaction volume was 1.0 ml. The $K_m$ and $V_{max}$ of free and phage-enzyme were measured with substrate concentrations between 0.2 $\mu$M and 1 mM. The kinetic parameters were determined by non-linear regression analysis of the data (EZ-Fit version 5.03; Perella Scientific, Amherst, NH, U.S.A.). In all cases hydrolysis of the substrate in the controls was negligible.

## RESULTS

### Construction and analysis of penicillin G acylase phagemids

The penicillin G acylase gene was amplified and modified using the primers coding for the *Sfi*I and *Not*I restriction sites. Only by using a PCR programme with a low annealing temperature and a low extension temperature was amplification of the DNA fragment obtained. The PCR fragment was digested and inserted in pCANTAB 5E. In pPGA-E-g3p the original g3p maturation site, SHS (one-letter amino acid codes), had been modified to AAQPAMA to resemble the consensus better [19]. This construct thus comprises the DNA coding for the modified g3p signal peptide, for the $\alpha$-subunit, the internal peptide and the $\beta$-subunit from penicillin G acylase, for the E-tag peptide and for the coding sequence for residues 3–406 of the g3p coat protein (pPGA-E-g3p; Figures 1A and 1B). We ascertained by inspection of the three-dimensional structure of the penicillin G acylase from *E. coli* [13] (the penicillin G acylase from *E. coli* has a 64% amino acid similarity to that from *A. faecalis* [9]) that the link between enzyme and g3p was properly positioned and was sufficiently long and flexible to allow the folding of the two separate proteins.

In a second construct the E-tag was replaced by a sequence coding for a collagenase-sensitive site and six consecutive histidine residues (pPGA-H-g3p, Figure 1B). The latter sequence was inserted to be used for large-scale isolation of the penicillin G acylase and to study the accessibility of a small tag located between the penicillin G acylase and the g3p coat protein (see below).

### Production of *A. faecalis* penicillin G acylase in *E. coli* by different phagemids

We tested whether the constructs pPGA-E-g3p and pPGA-H-g3p were able to synthesize an active penicillin G acylase in *E. coli*. The plasmids were introduced into cells (*E. coli* HB2151) that do not suppress the amber codon located between the penicillin G acylase and the coat protein. HB2151 cells were grown and the lac promoter was induced with IPTG. Cells containing either pPGA-E-g3p or pPGA-H-g3p were expected to yield the soluble, non-g3p-fused penicillin G acylase. The penicillin G acylase from pPGA-E-g3p has the E-tag at its C-terminus; the pPGA-H-g3p plasmid expresses the $His_6$ tag.

The cell-free extract, the periplasmic fraction and the growth medium from cells transformed with either the pPGA-E-g3p or the pPGA-H-g3p plasmid were tested for penicillin G acylase activity. All fractions collected from cells harbouring the pPGA-g3p plasmid yielded penicillin G acylase activity, whereas those from control cells did not. This demonstrates that the *A. faecalis* enzyme can be expressed in *E. coli*. Moreover, the modified g3p signal sequence from phage fd was apparently able to direct the immature penicillin G acylase polypeptide to the periplasm to be processed into its mature $\alpha$–$\beta$ heterodimeric form.

The enzyme activity differed in each cell fraction that was collected. The specific activity (units of penicillin G acylase activity per mg of total protein) was always highest in the periplasmic fraction. The value depended on the growth conditions and varied between 0.04 and 1.6 units/mg of protein. The ratio of the specific activities in the various cell fractions was always similar (periplasm to cell-free extract to medium, 10:2:1). This result is in accordance with the fact that maturation occurs in the periplasmic space. We attribute the presence of enzyme activity in the medium to cell lysis.

The expression of the $His_6$ tag at the C-terminus of the pPGA-H-g3p penicillin G acylase was tested by the ability to bind such a modified protein on $Ni^{2+}$-agarose. Penicillin G acylase activity (approx. 1 unit/ml), either purified or in a crude extract from HB2151 cells, was incubated with the $Ni^{2+}$-agarose in a phosphate/NaCl buffer. After several washing steps the bound material was eluted with imidazole. Only the eluate of the cell-free extract from pPGA-H-g3p cells showed enzyme activity; in the enzyme preparation from the construct lacking the $His_6$ tag (pPGA-E-g3p), all activity remained in the supernatant (results not shown). Thus $Ni^{2+}$ binding can be used to affinity-purify the penicillin G acylase equipped with a C-terminal $His_6$ tag.

### Phage display of penicillin G acylase: rescue, activity and immunostaining

*E. coli* TG1 cells suppress the TAG codon to form a fusion protein. TG1 cells with either phagemid pPGA-E-g3p or pPGA-

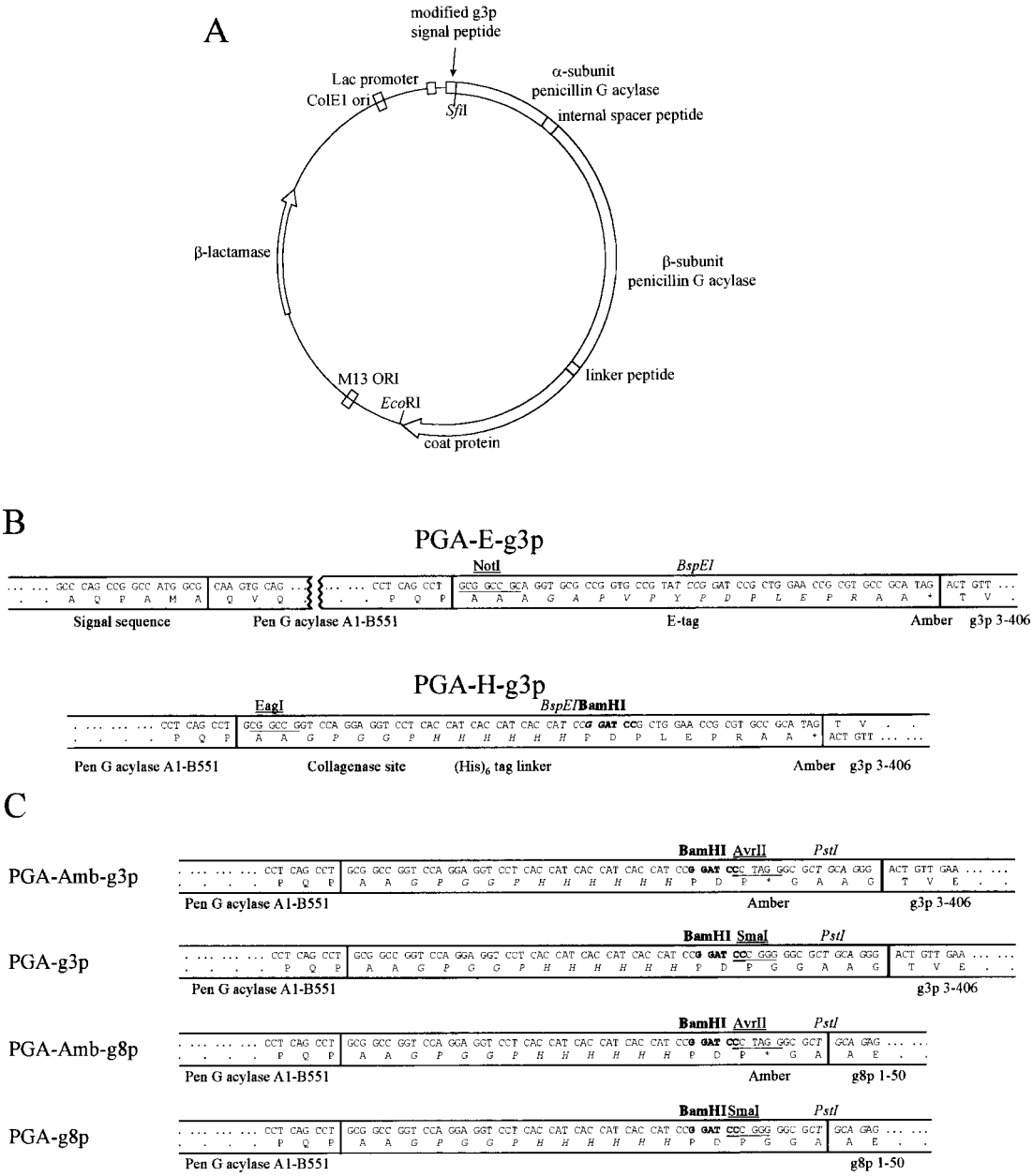


**Figure 1 Phagemids and the specific linker sequences used to display penicillin G acylase on phage fd**

(**A**) General layout of the phagemid, showing penicillin G acylase display vectors. (**B**) Composition of the linker peptide between the enzyme and the fd coat protein. (**C**) Molecular structure of the phagemids used to study the display dependence on coat protein type and nonsense codon suppression, showing linker regions for comparison of the display frequency.

H-g3p were superinfected with phage M13KO7. The cells were grown overnight in the presence of ampicillin and kanamycin. Phages thus produced would be expected contain both wild-type g3p and g3p fused to penicillin G acylase; the DNA inside the phage would be mostly from the phagemid. After removal of the cells by centrifugation, phages were harvested by precipitation and filter-sterilization.

PGA-E-g3p, PGA-H-g3p and control (M13KO7) phages were tested in the NIPAB hydrolysis assay. Both penicillin G acylase phage preparations hydrolysed the substrate, indicating that they displayed penicillin G acylase activity. Typically 0.5–2 m-units of enzyme activity was measured per ml of phage suspension. Thus penicillin G acylase could be displayed in an active manner via the g3p coat protein on the surface of the phage. This implies that the presence of the g3p coat protein attached to the penicillin G acylase polypeptide did not prevent its periplasmic maturation into an active penicillin G acylase; it also means that the presence of the 86 kDa heterodimer did not prevent the integration of the coat protein into the phage.

The presence of penicillin G acylase on the surface of the phage was confirmed by immunostaining the native phage particles with antibodies against the penicillin G acylase. Phagemids were run on an agarose gel. If penicillin G acylase and the phages were linked, the DNA, the phage protein and the penicillin G acylase would migrate together. The electrophoretic pattern of the phages on agarose gel is presented in Figure 2.

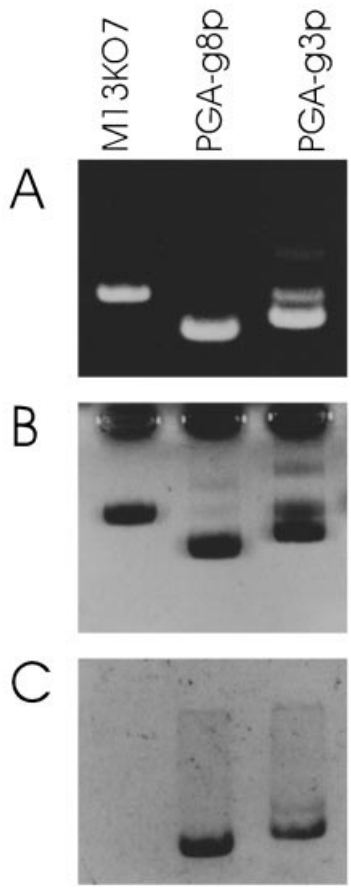


**Figure 2 Electrophoresis of whole phage particles on agarose**

After electrophoresis, phage particles were stained for DNA (**A**), protein (**B**) or penicillin G acylase (**C**). Lane 1, helper phage (M13KO7); lane 2, PGA-g8p phagemid; lane 3, PGA-g3p phagemid.

Various phage suspensions were loaded on the gel: the helper phage (M13KO7), phagemid PGA-g8p (see below) and phagemid PGA-g3p. The DNA was stained with ethidium bromide after disrupting the protein coat of the phage particles by alkaline solution (Figure 2A). Obviously the phages migrated according to the size of their DNA. Phagemids PGA-g3p (6860 nt) and PGA-g8p (5795 nt) ran faster than phage M13KO7 (8700 nt). The higher bands in the PGA-g3p lane probably represent phage particles with more phagemid DNA copies (polyphage).

Protein staining of the agarose gel yielded a similar pattern, indicating that the phages migrated as complete particles (Figure 2B). Phages were also blotted to nitrocellulose. Immunostaining with rabbit antibodies against *A. faecalis* penicillin G acylase resulted in bands for the phages attached to penicillin G acylase only (Figure 2C). Thus these phage particles, but not the control phages, displayed penicillin G acylase on their surface.

In another experiment we checked whether penicillin G acylase on the phage surface was indeed present as an enzyme–g3p fusion protein. Soluble enzyme and phages were loaded for SDS/PAGE under reducing conditions (Figure 3, left panel). The number of free enzyme molecules was approx. $10^{12}$, as determined by protein content and enzyme activity. Approx. $2.5 \times 10^8$ infective particles (PGA-g3p), as determined by titration, were loaded in a second lane. Loading more phage particles led to distortion of the electrophoretic pattern. After the run, protein was blotted to nitrocellulose and immunostained with antibodies against penicillin G acylase. The free enzyme showed two intense bands: the $\beta$ subunit (63 kDa) and the $\alpha$ subunit (23 kDa). The migration of the $\alpha$ and the $\beta$ subunits corresponded to apparent molecular masses of 29 and 63 kDa respectively. These observations are in accordance with previous results [9].

Phage proteins showed a double band at a molecular mass of more than 100 kDa. We attribute both bands to fusion proteins between $\beta$ subunit and g3p. There was also a faint band at 50 kDa, representing a degradation product that could also be detected in minute amounts in purified penicillin G acylase (results not shown).

The same phage particles displaying penicillin G acylase and M13KO7 control phages were also loaded on a 12.5 % (w/v) denaturing and reducing polyacrylamide gel, transferred to nitrocellulose and stained with antibodies against g3p (Figure 3, right panel). The same double band is visible in the lanes with enzyme-displaying phages. The molecular mass corresponds roughly to that calculated for the $\beta$ subunit–g3p fusion of 108 kDa. Therefore the same bands were detected with either the anti-(penicillin G acylase) or the anti-g3p antibody. In the lane containing the enzyme-displaying phages and in that containing the control phages, the non-fused g3p is visible at an apparent molecular mass of 65 kDa. Both the g3p and the fusion product show up as a mixture of two proteins. An almost identical pattern was explained previously in terms of proteolytic cleavage of the g3p [20].

### Kinetics of phage-displayed penicillin G acylase

Phages rescued from TG1 cells with pPGA phagemid display active penicillin G acylase on their surface. The fusion of the enzyme with the coat protein and the incorporation into the phage do not prevent the enzyme from being active. However, the behaviour of the enzyme, e.g. its $K_m$, might be altered by structural changes in the protein due to the attachment to the 42 kDa g3p protein. Measurement of the steady-state NIPAB hydrolysis by the phage-bound penicillin G acylase showed that the $K_m$ of the enzyme had not changed (Table 1). This indicates that the kinetic behaviour of the phage-displayed enzyme is similar to that of the free, soluble enzyme. The maximum activities ($V_{max}$) of the soluble enzyme and the phage-bound enzyme were also determined. The catalytic-centre activity of the purified, soluble enzyme was 50 $s^{-1}$, as calculated from the $V_{max}$ (34.7 units/mg) and the molecular mass (86 kDa). The catalytic-centre activity of a phage particle containing an unknown number of enzyme molecules was 6–20 $s^{-1}$, from a $V_{max}$ of $(1–6) \times 10^{-2}$ units/mg and the molecular mass of the phage of $2 \times 10^7$ Da (see the calculation in Table 2). Thus, on average, approx. 6–30 enzyme copies were actively displayed on the surface of every 100 phage particles, depending on the construct used and the phage rescue conditions. The average number of enzyme molecules per phage particle can also be calculated from the number of phage particles counted by their ability to infect bacteria (colony-forming units); using this number, both the value of the catalytic-centre activity per phage particle and the display level that can be calculated (see Table 2) become higher. Therefore the given display levels represent a lower limit.

### Roles of the phage coat protein and the amber stop codon in the yield of phage-displayed penicillin G acylase

The filamentous phage fd contains three to five copies of the g3p protein. Thus a maximum of five enzyme copies per phage can be

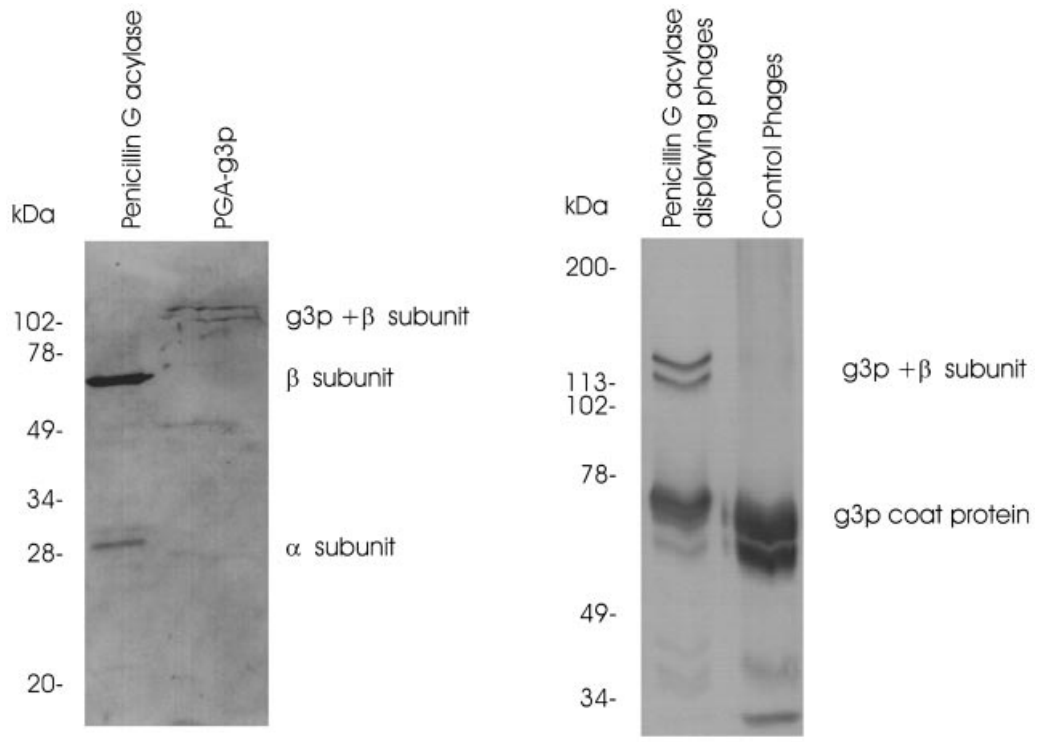


**Figure 3 SDS/PAGE patterns of phagemids displaying penicillin G acylase**

Left panel: soluble penicillin G acylase and phages on 10% (w/v) polyacrylamide. Lane 1, approx. $10^{12}$ penicillin G acylase molecules; lane 2, $2.5 \times 10^8$ colony-forming phagemids displaying penicillin G acylase (PGA-g3p). Immunostaining was with a rabbit antiserum against penicillin G acylase. Right panel: phagemids displaying penicillin G acylase and helper phages on 12.5% (w/v) polyacrylamide. Lane 1, approx. $2.5 \times 10^8$ phagemids displaying penicillin G acylase (PGA-g3p); lane 2, approx. $2.5 \times 10^8$ colony-forming helper phages (M13KO7). Immunostaining was with mouse monoclonal antibodies against g3p. The positions of molecular mass markers are indicated at the left of each panel.

**Table 1 Enzyme kinetics of penicillin G acylase from *A. faecalis***

Note that the $V_{max}$ of the phages varied between batches; the result of one phage rescue is shown here. The $K_m$ was independent of the batch. Values of $K_m$ and $V_{max}$ are given $\pm$S.E.M. (derived using non-linear regression analysis).

| Enzyme type | $V_{max}$ (units/mg) | $K_m$ ($\mu$M) |
|---|---|---|
| Soluble protein | $34.7 \pm 0.5$ | $4.4 \pm 0.3$ |
| Phage enzyme (coupled to g3p) | $1.9 \times 10^{-2}$ | $4.6 \pm 0.6$ |
| Phage enzyme (coupled to g8p) | $5.8 \times 10^{-2}$ | $3.6 \pm 0.3$ |

attained. However, the phage has approx. 45 copies of the major coat protein (g8p) per 100 nt of genetic information [16]. Thus the phagemids in this study have 2500–3000 g8p copies; coupling the enzyme to g8p might have the advantage of yielding a considerably higher display level, facilitating the detection and analysis of penicillin G acylase activity.

A second method of increasing the amount of displayed enzyme could be the removal of the amber stop codon between the penicillin G acylase gene and the g3p gene (Figure 1). Then only fusion products would be produced.

We constructed the plasmids representing the four possible combinations and compared their titre, display level and activity (Figure 1C and Table 2). In all cases the enzyme was properly expressed and folded. Even the fusion of the 86 kDa enzyme with the 5.5 kDa g8p allowed proper folding and maturation of the penicillin G acylase because we observed that this fusion protein was incorporated into the phage (Figure 2). Both modifications improved the display level: amber suppression release doubled the display factor, whereas g8p coupling tripled it (Table 2). The improvements were not as high as expected theoretically (50-fold and 500-fold respectively), indicating that the amount of enzyme displayed on the phage was limited by other factors.

### Immobilization of phages: inaccessibility of the $His_6$ tag

Constructs of penicillin G acylase and coat protein were equipped with a hexahistidine tag to facilitate the purification of recombinant penicillin G acylase molecules by adsorption on $Ni^{2+}$-NTA agarose. They were designed so that soluble protein would display the tag at its C-terminus, as described above. The soluble enzyme could be purified in an active form by binding it to the $Ni^{2+}$-NTA agarose. This result indicates that the tag was sufficiently exposed. Phages bearing the $His_6$ tag between the 86 kDa enzyme and the 47 (or 5.5) kDa coat protein were found not to interact with the $Ni^{2+}$-NTA agarose, indicating that in that case the tag was not sufficiently exposed to allow binding.

Interestingly, incubation of the phage suspension with the $Ni^{2+}$-NTA agarose column material did not at all decrease the enzyme activity in that suspension. Because free enzyme bound the $Ni^{2+}$ but fusion protein did not, the phage suspension did not contain any soluble, $His_6$-tagged penicillin G acylase; this again demonstrates the phage-linked position of the enzyme.

**Table 2 Effect of the coat protein and the linkage of the enzyme–coat fusion on phage-linked penicillin G acylase activity**

Note that the results originate from one experiment: the four different phages were isolated simultaneously. Numbers of phage particles were either determined by titration [colony-forming units (cfu)] or were calculated after protein measurement of the phage suspension (protein). The catalytic-centre activity was calculated as the number of NIPAB molecules hydrolysed per (phage) particle in 1 s. The catalytic-centre activity of the free enzyme was used to calculate the display level on phages as follows. For free penicillin G acylase, $V = 34.7$ units/mg; molecular mass $= 86 \times 10^3$ Da. So the catalytic centre activity was $34.7 \times 10^{-6}$ mol/min per mg $\times (60)^{-1}$ min/s $\times 86 \times 10^6$ mg/mol $= 50$ s$^{-1}$. For penicillin G acylase on phage linked to g3p, $V = 1.0 \times 10^{-2}$ units/mg; molecular mass $= 20 \times 10^6$ Da. The catalytic-centre activity of a phage particle was $1.0 \times 10^{-8}$ mol/min per mg $\times (60)^{-1}$ min/s $\times 20 \times 10^9$ mg/mol $= 3$ s$^{-1}$. For the calculation of the number of enzyme molecules per phage particle, the number of phages was based on the protein content of the solution.

| Coat protein | Amber | Activity phage suspension (m-units/mg) | $10^{-12} \times$ Number of phage particles | | Catalytic-centre activity per phage particle (s$^{-1}$) | | Enzyme molecules per phage particle |
|---|---|---|---|---|---|---|---|
| | | | cfu | Protein | cfu | Protein | |
| g3p | + | 10 | 15 | 71 | 14 | 3 | 0.06 |
| g3p | − | 40 | 8 | 53 | 40 | 6 | 0.12 |
| g8p | + | 36 | 14 | 72 | 49 | 9 | 0.19 |
| g8p | − | 57 | 21 | 78 | 53 | 15 | 0.30 |
| Soluble enzyme | | $34.7 \times 10^3$ | | | | 50 | |

## DISCUSSION

We have fused the full penicillin G acylase precursor, including its natural internal peptide, to the phage coat protein to obtain phage display of the enzyme.

The observation of enzymically active penicillin acylase being produced by cells as a fusion indicates that the 133 kDa prepro-fusion protein is properly transported to the periplasm and processed into the $\alpha$ and $\beta$ subunits. Native phage electrophoresis on agarose and histidine-affinity chromatography also show that the enzyme is physically bound to the phage particle. The immunostaining experiments with antibodies against either the enzyme or the minor coat protein confirm that the penicillin acylase is produced as a fusion product with g3p. This is the first example of a heterodimeric enzyme functionally displayed on phage. Furthermore, it shows that, in a fusion with g3p, processing of a single large polypeptide chain into its subunits can still take place and that assembly of these subunits into phage particles occurs. These results imply that the transport of the folded enzyme dimer fused to g3p across the outer membrane probably occurs via the phage extrusion-specific g4p multimer [21].

The substrate affinity of the phage-immobilized enzyme is identical with that of soluble penicillin acylase, indicating the enzyme's proper folding and action. Relating the activity measurements to either phage titration or quantitative protein measurements demonstrates that 1–30 % of the phages display the enzyme.

Competition between wild-type and fusion coat protein results in a limited incorporation of fusion protein in the phage particle because we use the phagemid system. We have studied two means of increasing the enzyme copy number on the phage.

The first was by addressing the presence of the amber codon that is located between the enzyme and the coat protein. On the basis of suppression frequencies, theoretically 2 % of the enzyme produced in the construct (PGA-Amb-g3p) will be in the form of a fusion protein; the other 98 % will be in the free form [22]. Thus by replacing this codon with a sense codon one would expect a 50-fold increase in the fusion product yield. The increase that we observed was only 2-fold.

The second was g8p display: if the g3p copy number on the phage limits the display level, we should be able to increase this level considerably by coupling the enzyme to the major coat protein g8p, which is present in about 2500 copies per phage particle. However, in our experiments less than 1 % of the g8p on the phage was present as a fusion to penicillin G acylase. The copy number of the coat protein therefore augments the absolute display level (we did observe a 3-fold increase), but not to the extent that was expected theoretically.

It is therefore not possible to obtain polyvalent display of the penicillin G acylase merely by increasing the production of fusion protein. We speculate that another factor prevents multi-copy display of enzyme: the size of the gated channel in the outer membrane formed by the secretin g4p has been estimated to be 65–80 Å [21,23], which implies that the 133 kDa fusion protein of penicillin G acylase with g3p (the diameter of the enzyme is already 50 Å) barely fits the outer membrane export channel. Thus extrusion of the displaying phage is possible only when the enzyme present on the phage surface does not increase the effective diameter of the growing phage, which is 65 Å [23]. Polyvalent display of bulky proteins such as penicillin G acylase might therefore not be possible because of size limitations of the g4p export channel. The size limitations of the g4p export channel may also hamper the polyvalent display of g8p fusions.

Interestingly, the size of the fusion of penicillin G acylase with g3p (113 kDa) is very close to the 117 kDa *Klebsiella pneumoniae* pullulanase, which is exported by a similar gated outer-membrane channel formed by the homologous secretin *pulD* [24].

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