

# The secondary cell wall polysaccharide of *Bacillus anthracis* provides the specific binding ligand for the C-terminal cell wall-binding domain of two phage endolysins, PlyL and PlyG

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Endolysins are bacteriophage enzymes that lyse their bacterial host for phage progeny release. They commonly contain an N-terminal catalytic domain that hydrolyzes bacterial peptidoglycan (PG) and a C-terminal cell wall-binding domain (CBD) that confers enzyme localization to the PG substrate. Two endolysins, phage lysin L (PlyL) and phage lysin G (PlyG), are specific for *Bacillus anthracis*. To date, the cell wall ligands for their C-terminal CBD have not been identified. We recently described structures for a number of secondary cell wall polysaccharides (SCWPs) from *B. anthracis* and *B. cereus* strains. They are covalently bound to the PG and are comprised of a -ManNAc-GlcNAc-HexNAc- backbone with various galactosyl or glucosyl substitutions. Surface plasmon resonance (SPR) showed that the endolysins PlyL and PlyG bind to the SCWP from *B. anthracis* (SCWP<sub>Ba</sub>) with high affinity (i.e. in the  $\mu\text{M}$  range with dissociation constants ranging from  $0.81 \times 10^{-6}$  to  $7.51 \times 10^{-6}$  M). In addition, the PlyL and PlyG SCWP<sub>Ba</sub> binding sites reside with their C-terminal domains. The dissociation constants for the interactions of these endolysins and their derived C-terminal domains with the SCWP<sub>Ba</sub> were in the range reported for other protein-carbohydrate interactions. Our findings show that the SCWP<sub>Ba</sub> is the ligand that confers PlyL and PlyG lysin binding and localization to the PG. PlyL and PlyG also bound the SCWP from *B. cereus* G9241 with comparable affinities to SCWP<sub>Ba</sub>. No detectable binding

was found to the SCWPs from *B. cereus* ATCC (American Type Culture Collection) 10987 and ATCC 14579, thus demonstrating specificity of lysin binding to SCWPs.

**Keywords:** *Bacillus anthracis* / bacteriophage / endolysin / polysaccharide / secondary cell wall polymer

## Introduction

*Bacillus anthracis* is the etiologic agent of anthrax, a disease primarily of herbivores, although humans can also be infected. Regardless of *B. anthracis*' route of entry (cutaneous, gastrointestinal or inhalation), systemic anthrax has a high case-fatality ratio. *Bacillus anthracis* has long been recognized as a potential biological weapon (Hilleman 2002; Baillie 2005). In particular, since the anthrax bioterrorism events in 2001, there has been a renewed interest in effective diagnostic tools and medical countermeasures (Russell 2007).

Endolysins are bacteriophage-encoded enzymes capable of hydrolyzing the peptidoglycan (PG) of the cell wall murein layer. Because of this activity, endolysins have also been termed phage lysozymes, lysins or muralytic or mureolytic enzymes. They are considered potent antibacterials and a potential alternative to antibiotics for the control of pathogenic bacteria, including *B. anthracis* and *B. cereus*. Endolysins are expressed intracellularly in phage-infected bacterial cells at the end of the phage lytic cycle during multiplication to release the phage progeny. Endolysins are characterized by their ability to target and cleave bonds of the PG thereby degrading the rigid PG murein layer, which leads to cell lysis and, in turn, to the release of the newly assembled virions (for review, see Loessner 2005).

Two well-characterized endolysins capable of lysing *B. anthracis* cells are phage lysin G (PlyG) and phage lysin L (PlyL) (Schuch et al. 2002; Low et al. 2005). The PlyG protein was initially identified in the  $\gamma$ -phage (Schuch et al. 2002). This phage, at first isolated as a variant of the original *Bacillus* strain W bacteriophage, was described as *B. anthracis*-specific (McCloy 1951; Brown and Cherry 1955). The PlyL protein, on the other hand, was isolated from a lysogenic copy of the  $\lambda$  Ba02-prophage integrated into the *B. anthracis* genome (Low et al. 2005). Investigations into the activity spectrum of these

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phages showed that the lytic activity of both enzymes is not strictly specific for the lysis of *B. anthracis* cells, since certain *B. cereus* strains closely related to *B. anthracis* are also lysed (e.g. strain *B. cereus* ATCC 4342 by PlyG; Schuch et al. 2002; Low et al. 2005, 2011), but these strains are exceptions. Of applied interest is the fact that when added to cells extracellularly these endolysins retain their lytic activity, even in the presence of capsular S-layer proteins, showing that the PG substrate is accessible to the endolysins to some degree even in the presence of S-layer proteins (Schuch et al. 2002; Fischetti 2010; Low et al. 2011).

The endolysin molecular architecture is dictated by two basic functions: substrate recognition and hydrolysis (Loessner 2005). As with other endolysins, these two functions are reflected in the modular architecture of PlyL and PlyG.

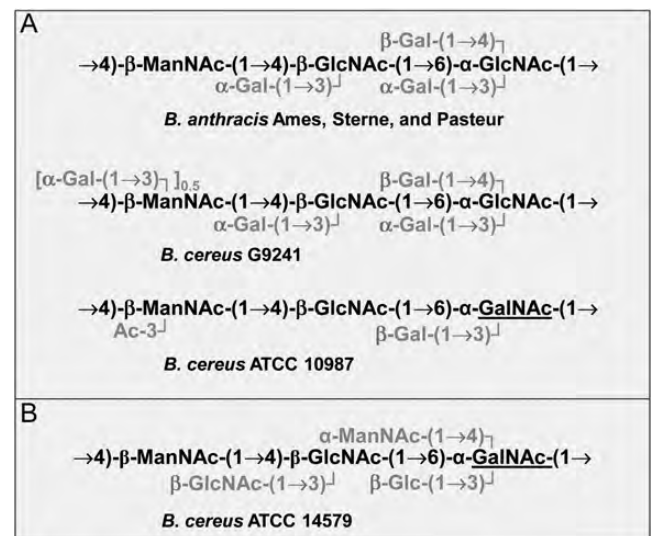
The C-terminal domain of these proteins contain a cell wall-binding domain (CBD) conferring specificity for a cell wall component; their CBDs are not of a well-characterized type and their receptor ligands have not yet been described. In the N-terminal domain of these proteins, they harbor the catalytic domain for the hydrolysis of PG (Schuch et al. 2002; Low et al. 2005, 2011; Fujinami et al. 2007; Kikkawa et al. 2007, 2008). The related functions of these enzyme domains are reflected in the homology of their amino acid sequences, which show 60% identity in the C-terminal region and 93% in the N-terminal region of the protein (Low et al. 2005). The less pronounced homology of the C-terminal domains could indicate that the C-terminal CBDs of both enzymes bind to a related structure in the *B. anthracis* cell wall, but with somewhat different binding properties. On the other hand, the high homology of the N-terminal regions corroborates the *N*-acetylmuramoyl-L-alanine amidase activity suggested for both catalytic domains (Schuch et al. 2002; Low et al. 2005; Kikkawa et al. 2008).

Classical lysins require the CBD to bind to a cell wall component and orient the catalytic domain of the enzyme to the PG. In this regard, PlyG and PlyL may show a difference. It has been reported that PlyG behaves as a classical PG-degrading enzyme with its CBD binding to the cell wall ligand, a precondition for reactivity (Schuch et al. 2002, Kikkawa et al. 2008). However, somewhat similar to what had previously been observed for a *B. anthracis*-encoded PG hydrolyzing autolysin (Mesnage and Fouet 2002), a recent paper reports lytic activity and non-classical behavior for the catalytic domain of PlyG, i.e. lytic activity in the absence of the CBD (Low et al. 2011). This contradiction to the older PlyG findings is as yet unexplained. For PlyL, non-classical behavior has also been reported with the isolated N-terminal region of PlyL retaining PG-degrading activity without an attached CBD (Low et al. 2005, 2011). It has been reported for both PlyL and PlyG that the removal of the C-terminal domain of the full-length proteins decreases the reactivity for *B. anthracis* strains and also broadens the reactivity to more *Bacillus* species (Low et al. 2005, 2011). Hence, either for efficient lysis or cell lysis specificity of host cells, both PlyG and PlyL require their CBD to bind to a cell wall ligand. To date, this cell wall ligand in the *B. anthracis* and *B. cereus* strains has not been identified.

Interest in *B. anthracis* carbohydrates has increased, since the discovery by Mesnage et al. (1999) that S-layer cell surface

proteins contain an S-layer homology (SLH) domain that non-covalently binds to a *B. anthracis* cell wall carbohydrate and this way anchors the proteins to the bacterial vegetative cell wall (Mesnage et al. 1999, 2000; Fouet 2009; Kern and Schneewind 2010). This carbohydrate, categorized as a non-classical, secondary cell wall polysaccharide (SCWP; Schäffer and Messner 2005), is thought to be covalently attached to the PG through a phosphate group at the reducing end of the polysaccharide (Leoff, Saile, et al. 2008; Kern et al. 2010; Schneewind and Messiakas 2012) and can be released from the cell wall by treatment with cold aqueous hydrofluoric acid. We recently used this procedure to isolate and determine the structures of the SCWPs from *B. anthracis* Sterne strains 34F<sub>2</sub> and 7702 (both strains have a S-layer, but lack virulence plasmid pXO2 coding for the poly-γ-glutamate capsule), from *B. anthracis* Pasteur (this strain has a S-layer, but lacks virulence plasmid pXO1), and the fully equipped *B. anthracis* Ames strain (Choudhury et al. 2006), as well as from two *B. cereus* strains closely related to *B. anthracis* (i.e. *B. cereus* G9241, isolated from a case of severe pneumonia in humans, and *B. cereus* ATCC 10987, isolated from dairy products; Leoff, Choudhury, et al. 2008; Forsberg et al. 2011, 2012). In addition, recently Candela et al. (2011) have reported the SCWP structure from the more distantly related type strain, *B. cereus* ATCC 14579.

The structures of the SCWPs found in the vegetative cell walls of these strains are shown in Figure 1 (compiled from



**Fig. 1.** (A) The SCWPs from *B. anthracis* and the indicated *B. cereus* strains with a related glycosyl backbone structure. The glycosyl backbones of these SCWPs all consist of amino sugar shown in black, which are decorated by glycosyl or acetyl residues shown in gray. The GalNAc of the *B. cereus* ATCC 10987 SCWP backbone that is different from the *B. anthracis* SCWP is underlined. Strain *B. cereus* G9241 is a clinical isolate from a case of severe pneumonia, and *B. cereus* strain ATCC 10987 does not cause severe or fatal disease. (B) The SCWP of the type strain *B. cereus* ATCC 14579 differs significantly. Its trisaccharide backbone is the same as that for *B. cereus* ATCC 10987; however, the terminal glycosyl residues also include aminoglycosyl residues and Glc instead of Gal or acetyl substituents (adopted from Leoff, Saile, et al. 2008; Candela et al. 2011).

Choudhury et al. 2006; Leoff, Choudhury, et al. 2008; Candela et al. 2011; Forsberg et al. 2011). In *B. anthracis* strains examined to date, the SCWPs (SCWP<sub>Ba</sub>) have the same structure comprised of an amino sugar backbone of  $\rightarrow 4$ - $\beta$ -D-ManpNAc-(1 $\rightarrow$ 4)- $\beta$ -D-GlcpNAc-(1 $\rightarrow$ 6)- $\alpha$ -D-GlcpNAc-(1 $\rightarrow$  in which the  $\alpha$ -GlcNAc residue was substituted with  $\alpha$ -Gal and  $\beta$ -Gal at O3 and O4, respectively, and the  $\beta$ -GlcNAc substituted with  $\alpha$ -Gal at O3 (Choudhury et al. 2006). This SCWP<sub>Ba</sub> structural theme is conserved in strain *B. cereus* G9241 SCWP (SCWP<sub>BcG9241</sub>), but contains an additional  $\alpha$ -Gal substitution on  $\sim$ 50% of the  $\beta$ -ManpNAc residues at O3 (Forsberg et al. 2011). In comparison, the SCWP from the closely related strain *B. cereus* ATCC 10987 (SCWP<sub>10987</sub>) consists of a  $\rightarrow 4$ - $\beta$ -D-ManpNAc-(1 $\rightarrow$ 4)- $\beta$ -D-GlcpNAc-(1 $\rightarrow$ 6)- $\alpha$ -GalpNAc-(1 $\rightarrow$  backbone in which the  $\alpha$ -GalNAc is substituted at O3 with a  $\beta$ -Gal residue and the  $\beta$ -ManNAc is acetylated at O3 (Leoff, Choudhury, et al. 2008). From these different SCWP structures identified so far (Figure 1), a common structural theme has emerged consisting of a -ManNAc-GlcNAc-HexNAc- backbone that is substituted with terminal galactosyl (Gal) or glucosyl (Glc) residues or non-carbohydrate substituents such as acetyl, amino or pyruvyl groups (Figure 1A; Choudhury et al. 2006; Leoff, Saile, et al. 2008; Forsberg et al. 2011, 2012). The SCWP from the type strain *B. cereus* ATCC 14579 (SCWP<sub>14579</sub>) presents a significantly different structural motif in which the -ManNAc-GlcNAc-HexNAc- backbone is substituted with terminal GlcNAc, ManNAc and Glc residues (Figure 1B; Candela et al. 2011). Recent studies have provided sufficient evidence that the SCWPs, compared with other bacterial surface carbohydrates such as exopolysaccharides, are relatively small polysaccharides (their molecular weights ranging from  $\sim$ 10,000 to 20,000 Da depending on the strain) that display rather minimal size heterogeneity during size exclusion chromatography (Mesnage et al. 2000; Forsberg et al. 2011).

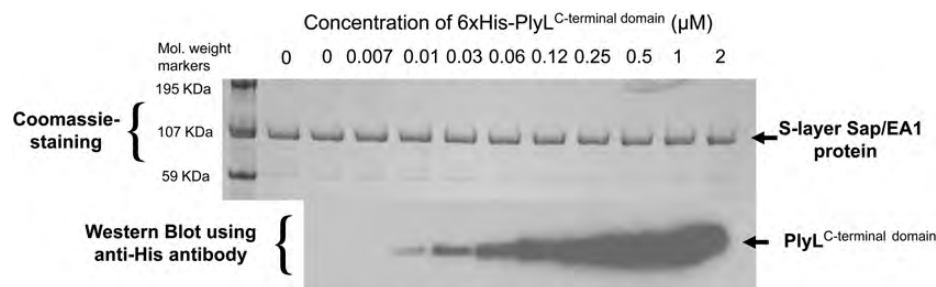
The attachment of these SCWPs to PG, together with the presence of their strain-specific as well as more general species-specific structural features, lead us to hypothesize that these polysaccharides could function as ligands for endolysin binding in the *B. anthracis* and *B. cereus* cell walls. Therefore,

we used SPR (Biacore, Piscataway, NJ, USA) in conjunction with full-length PlyL and PlyG proteins and their C- and N-terminal domains to investigate the binding affinities of these endolysins to SCWPs. The SCWPs from the different *B. anthracis* and *B. cereus* strains that went into this study were chosen because they are structurally related (Figure 1) and were used to test whether identical SCWPs (as in the case of the SCWPs from *B. anthracis* Sterne 34F<sub>2</sub> and 7702) display similar binding properties. We show here that the full-length PlyL and PlyG and their C-terminal domains bind to the SCWP<sub>Ba</sub> from *B. anthracis* but not to the SCWPs from the closely related *B. cereus* ATCC 10987 and the more distantly related type strain, *B. cereus* ATCC 14579. Interestingly, PlyG also bound to the SCWP from *B. cereus* G9241, an isolate from a case of severe pneumonia. Our findings support the model that the SCWP<sub>Ba</sub> is the ligand for the CBD of PlyL and PlyG and that this binding localizes and orients PlyL and PlyG, so they can carry out their lytic activity on the PG. This binding also accounts for the specificity of these endolysins.

## Results

### Lysin PlyL C-terminal domain binds to *B. anthracis* cells

Previous work showed PlyG's and PlyL's modular protein architecture is comprised of a catalytic N-terminal domain and a C-terminal CBD (Low et al. 2005; Kikkawa et al. 2008) and directly binds the PlyG C-terminal domain to *B. anthracis* cells (Fujinami et al. 2007). Binding of PlyL CBD to *B. anthracis* cells has only recently and as yet rather selectively been addressed (Low et al. 2011). To confirm and extend the observations on the binding ability of the PlyL C-terminal domain to whole *B. anthracis* cells, we performed a pull-down experiment with strain *B. anthracis* 34F<sub>2</sub> cells pre-exposed to increasing amounts of the PlyL C-terminal protein domain. As shown in Figure 2, gels loaded with pulled-down *B. anthracis* cells contained bound PlyL C-terminal domains. The amount of the C-terminal domain that was pulled down was dependent on the amount of the PlyL C-terminal domain used for pre-exposure and reached a



**Fig. 2.** Pull-down assay of the endolysin PlyL C-terminal domain using live *B. anthracis* Sterne 34F<sub>2</sub> cells. The top panel shows a Coomassie-stained PAGE gel of the samples analyzed. Equal loading of *B. anthracis* cells was confirmed making use of the prominent  $\sim$ 100 kDa band of the S-layer proteins Sap and EA1 (at 0  $\mu$ M PlyL C-terminal domain, the equal number of *B. anthracis* cells was analyzed without PlyL C-terminal domain pretreatment). The western blot at the bottom of the figure shows the increasing amounts of the pulled-down PlyL C-terminal domain bound to *B. anthracis* cells after pretreatment with increasing concentrations of the PlyL C-terminal domain. For that, tubes containing a cell suspension/endolysin protein mixture were placed on a rocking platform and incubated for 10 min. After incubation, the cells were pelleted and resuspended in phosphate-buffered saline, washed and mixed with an equal volume of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer, boiled and analyzed on SDS-PAGE gels. For western blots, the anti-His antibody and the Goat horseradish peroxidase conjugated anti-mouse antibody were used as the primary and the secondary antibodies, respectively (for more details, see *Experimental*).



maximum signal at  $\sim 0.5\text{--}1.0\ \mu\text{M}$ . This shows that the PlyL C-terminal domain binds to a ligand in the *B. anthracis* cell wall. The finding indicates that under the chosen experimental conditions either the number of binding sites on the cell surface is limited or the amounts of PlyL bound to the nitrocellulose sheet is saturable.

#### Ply binding studies

We used SPR to investigate SCWP–Ply interactions as it is a rapid and sensitive method to evaluate molecular affinities; the method is based solely on mass changes occurring during the interactions, allowing for the assessment of the interactions in real time and without the involvement of external labels, e.g. fluorophores, which can have modifying effects on the molecular interactions. This may be particularly relevant to this study, as the method avoids the increase in sample heterogeneity in the already size heterogeneous polysaccharide analyte samples. The full-length proteins as well as the C- and N-terminal domains of the PlyL and PlyG proteins were immobilized onto *N*-hydroxysuccinimide (NHS)-activated CM5 sensor chips and titrated with increasing amounts of SCWP isolated from different *B. cereus* group strains. Bulk refractions instigated by sample injections to the running buffer and non-specific binding were ameliorated with the help of a control cell pretreated with ethanolamine. Sensorgrams were evaluated using double referencing, global fitting and interaction models as indicated in figures and tables.

#### PlyL and PlyG bind to SCWP<sub>Ba</sub> from strains *B. anthracis* 34F<sub>2</sub> and 7702

To determine whether SCWP<sub>Ba</sub> is the ligand for PlyL and PlyG, we first measured the binding properties of the full-length PlyL and PlyG proteins using SPR. Figure 3 shows the BIAcore sensorgrams obtained with the immobilized proteins and increasing concentrations of the SCWP<sub>Ba</sub> analyte isolated from strains *B. anthracis* 34F<sub>2</sub> (SCWP<sub>Ba34F2</sub>) and 7702 (SCWP<sub>Ba7702</sub>). Both full-length proteins displayed strong affinities to isolated SCWP<sub>Ba</sub> from the two *B. anthracis* strains tested and sensorgrams shifted with increasing analyte concentrations to higher response unit (RU) values. For the calculation of the binding kinetic parameters, different binding models supplied by the BIAevaluation 4.1 software were evaluated. For the PlyL proteins, a two-state interaction model was found to be the best fit (Figures 3 and 4). In contrast, for the PlyG full-length protein using global fitting, binding curves did not fit well to the different models included in the BIAevaluation software. Furthermore, because equilibrium was not reached during the association phase, the direct use of Scatchard analysis to calculate the apparent equilibrium dissociation constant was not allowed. Instead, the association rate constants ( $k_a$ ) were derived from the association phase using the 1:1 Langmuir association ( $k_{\text{obs}}$ ) model and a secondary plot of  $k_{\text{obs}}$  vs analyte concentration (see inserts in Figures 3 and 4). The dissociation rate constant ( $k_d$ ) was calculated using the 1:1 Langmuir dissociation model, and the dissociation constant,  $K_D$ , was obtained from the ratio of  $k_d/k_a$  as described in the method section. The dissociation constants  $K_D$  calculated from these curves for PlyL and PlyG full length are in the  $\mu\text{M}$  range, i.e. with  $K_D$  values ranging from  $0.81 \times 10^{-6}$  to  $7.51 \times 10^{-6}$  M (Table I).

These  $K_D$  values are in the range for what has been reported for some carbohydrate protein binding, e.g. for “bacterial” lectin to carbohydrate binding (Imberty et al. 2005). However, for lysins, stronger binding affinities have also been reported, e.g. *Pseudomonas* endolysin binding to PG  $K_D = 3.4 \times 10^{-8}$  M (Briers et al. 2009), or for *Listeria monocytogenes* bacteriophage murein hydrolase binding to bacterial cell wall carbohydrates,  $K_D = 3.1 \times 10^{-9}$  M (Loessner et al. 2002). Also, from PlyL binding affinity to whole cells of *B. cereus* ATCC 4342, Low et al. (2011) estimated a  $K_D$  of  $8 \times 10^{-9}$  M, indicating a higher affinity of endolysins to whole cells as has been observed here with isolated SCWPs.

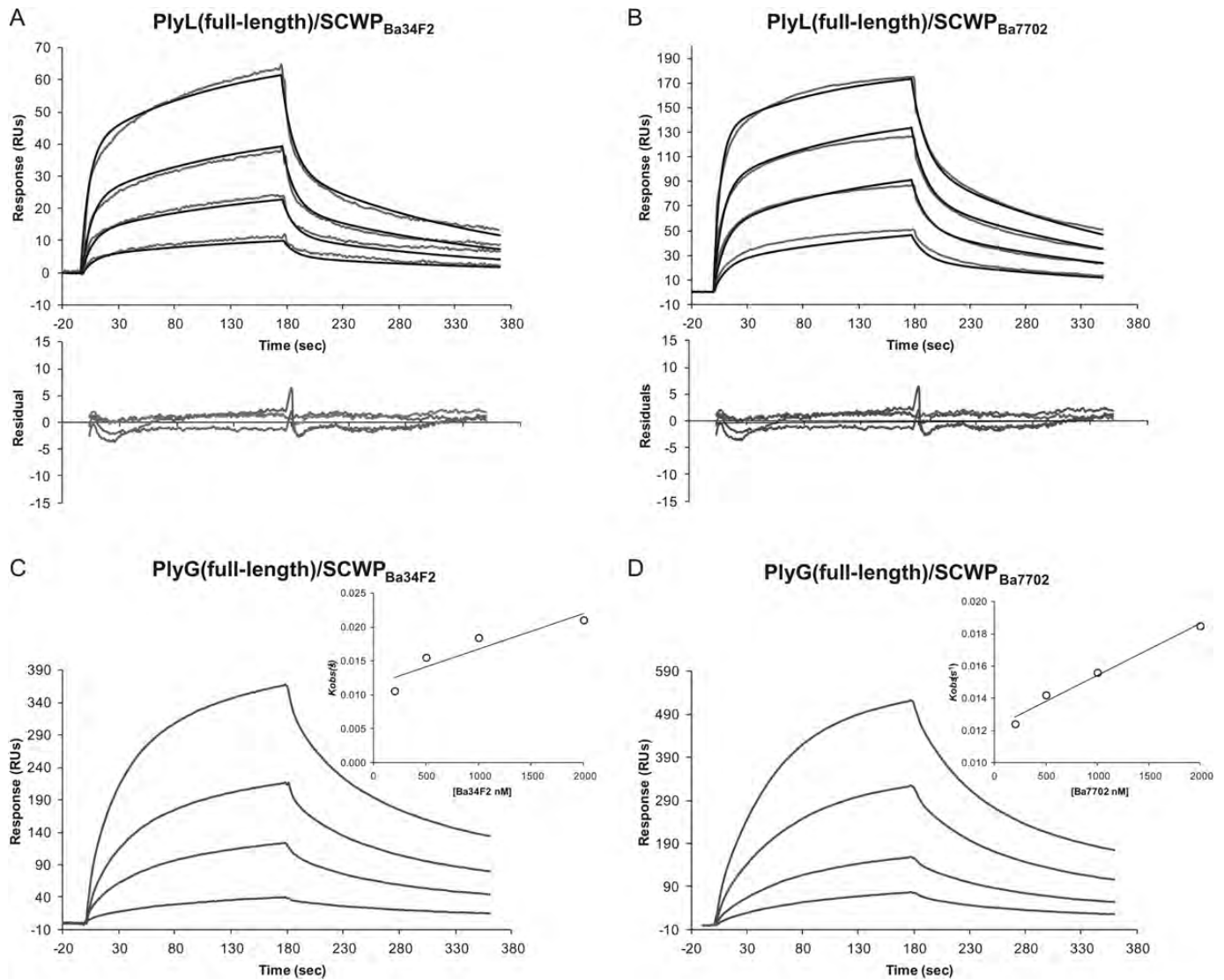
#### Binding of PlyL and PlyG C- and N-terminal domains to SCWP<sub>Ba34F2</sub> and SCWP<sub>Ba7702</sub>

We next determined whether the proposed modular structure of PlyL and PlyG with their N-terminal catalytic domain and C-terminal CBD is reflected in the abilities of the PlyL and PlyG C- and N-terminal domains to bind the SCWP<sub>Ba</sub>. Figure 4 shows the BIAcore sensorgrams obtained using the PlyL and PlyG C-terminal domains in conjunction with various concentrations of SCWP<sub>Ba34F2</sub> and SCWP<sub>Ba7702</sub>. The C-terminal domains from PlyL and PlyG bound both *B. anthracis* SCWP<sub>Ba</sub> samples in a concentration-dependent manner with increasing RU values similar to those for the respective full-length proteins (Figures 3 and 4). The binding affinities (Table I) between the PlyL C-terminal domains and both SCWP<sub>Ba</sub>, as shown by the  $K_D$  values, were in the micromolar range (i.e. from  $1.14 \times 10^{-6}$  to  $2.66 \times 10^{-6}$  M) and approximately in the range of the respective  $K_D$  values obtained with the PlyG C-terminal domain (i.e.  $0.81 \times 10^{-6}$  and  $0.62 \times 10^{-6}$  M), thus supporting the results with the full-length proteins that showed approximately similar affinities of PlyL and PlyG toward SCWP<sub>Ba</sub>.

The N-terminal domains from PlyL and PlyG did not bind to the SCWP<sub>Ba</sub>. Figure 5 shows an example of this result with regard to SCWP<sub>Ba34F2</sub> binding to the PlyL N-terminal domain. The same series of SCWP<sub>Ba34F2</sub> concentrations (0–2000 nM) showed no detectable binding to the PlyG N-terminal domain (data not shown). These results showed that the ability of PlyL and PlyG to bind the SCWP<sub>Ba</sub> resides in their C-terminal and not their N-terminal domain. Also noteworthy, the PG-degrading activity of PlyL and PlyG that has recently been reported and that is independent of the CBD (Low et al. 2005, 2011) does not involve binding of the N-terminal domain to the SCWP<sub>Ba</sub>, but seems to employ an alternative binding mechanism for CBD-independent PG binding and degrading.

#### Comparing the PlyL and PlyG affinity for SCWP<sub>Ba34F2</sub> and SCWP<sub>Ba7702</sub>

Since the SCWP<sub>Ba</sub> structures of *B. anthracis* 34F<sub>2</sub> and 7702 are identical with regard to the arrangement of their glycosyl residues and molecular weight (Choudhury et al. 2006; Leoff, Choudhury, et al. 2008), we expected them to show identical interactions with the Ply proteins. This is, indeed, what happened. The binding affinities of PlyL and PlyG full-length proteins to both of these SCWPs were about the same (Figure 3 and Table I). However, the overall comparison of the  $K_D$  values obtained with full-length and C-terminal domains of PlyL and PlyG seems to indicate a somewhat

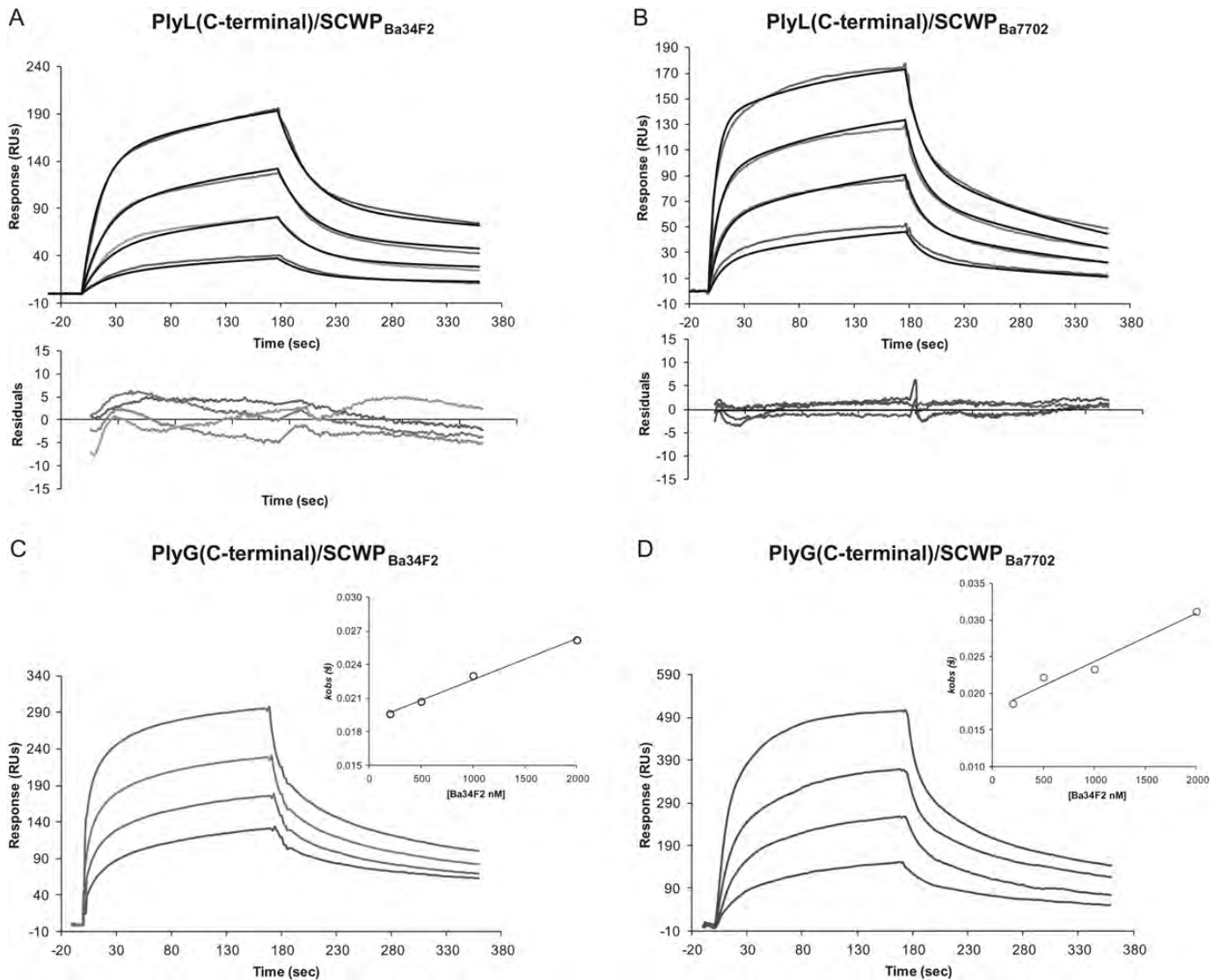


**Fig. 3.** Kinetics of the interaction of endolysins PlyL (full length) (A and B) and PlyG (full length) (C and D) with SCWP<sub>Ba</sub> samples isolated, as indicated, from *B. anthracis* Sterne strains 34F<sub>2</sub> and 7702. The SCWP concentrations used were from the top to the bottom: 2000, 1000, 500, 200 and 0 nM. The baseline response obtained with no (the 0 nM control) SCWP analyte has been subtracted from the binding curves and is not shown. For PlyL curves, a two-state interaction model gave the best fit (calculated and observed curves are superimposed with the residual curves obtained underneath); for PlyG, the association rate constants ( $k_a$ ) were determined from the slope of a plot of the observed association rate constant ( $k_{obs}$ ) as a function of the analyte concentration  $C$ , according to  $k_{obs} = k_a C + k_d$ . (the inserts) The dissociation rate constant ( $k_d$ ) was calculated using the 1:1 Langmuir dissociation model from which dissociation constant,  $K_D$  was obtained from the ratio of  $k_d/k_a$ . The SCWP samples were dissolved in HBS-EP buffer and were injected for 180 s at a flow rate of 10  $\mu$ L/min. After the dissociation phase, the chip surface was regenerated with an injection of glycine (pH 2.0), followed by a pulse of 1.5 M NaCl solution. Chips were reused as long as the RU response to test samples were reproducible (for more details, see *Experimental*).

stronger affinity of PlyG toward SCWP<sub>Ba</sub>, but it remains to be seen in future binding studies if that is indeed the case. In summary, our results indicate strong binding of PlyL and PlyG to both SCWP<sub>Ba</sub> samples with approximately the same binding affinity. Also, the fact that there is no significant difference in the binding affinities between PlyL and PlyG toward SCWP<sub>Ba7702</sub> and SCWP<sub>Ba34F2</sub> is consistent with our structural data which show that there is no structural difference between these two SCWP<sub>Ba</sub> (Choudhury et al. 2006; Leoff, Choudhury, et al. 2008; Forsberg et al. 2012).

#### *The specificity of PlyL and PlyG for SCWPs*

It is known that PlyG and PlyL are specific to *B. anthracis* strains and to a few closely related *B. cereus* strains (see *Introduction*). In order to determine if the interactions of these endolysins with the SCWPs could account for this specificity, we measured endolysin binding to a variety of recently characterized SCWPs from different *B. anthracis* and *B. cereus* strains (the SCWP structures are shown in Figure 1). The BIAcore sensorgrams in Figures 6 and 7 compare binding of the full-length proteins and their C-terminal domains to these SCWPs.



**Fig. 4.** BIAcore analysis of the interaction of SCWP<sub>Ba</sub> samples isolated from *B. anthracis* Sterne strains 34F<sub>2</sub> and 7702 with the immobilized C-terminal domain of endolysin PlyL (A and B) and PlyG (C and D). Further details, the experimental conditions and the concentrations of SCWPs were the same as in Figure 3.

With SCWP<sub>Bc14579</sub>, RU values obtained for both PlyL and PlyG full-length proteins indicated some weak interaction; however, after global fitting analysis, the kinetic parameters could not be determined. In addition, the PlyG C-terminal domain showed no binding at all with SCWP<sub>Bc14579</sub>, while the binding constant for the PlyL C-terminal domain could not be determined. Together, these results indicate some non-specific binding of the full-length proteins to SCWP<sub>Bc14579</sub>. Moreover, neither full-length PlyL and PlyG proteins nor their C-terminal domains bound SCWP<sub>Bc10987</sub> (Figures 6 and 7 and Table I).

In contrast, SCWP<sub>BcG9241</sub>, which shares close structural similarity with *B. anthracis* SCWP<sub>Ba</sub>, did bind to both full-length proteins. The binding of the full-length proteins to SCWP<sub>BcG9241</sub> was also reflected in the binding of their C-terminal domains to SCWP<sub>BcG9241</sub> (Figures 6 and 7). Both C-terminal domains showed higher binding affinities to SCWP<sub>BcG9241</sub> when compared with their full-length counterparts. However, the measured

sensorgrams indicate a difference in the binding affinities of PlyL and PlyG toward SCWP<sub>BcG9241</sub>. Although the PlyL full-length and C-terminal domain binding affinities to SCWP<sub>BcG9241</sub> were ~4–10-fold weaker compared with those with SCWP<sub>Ba</sub> (Table I), the binding affinity of PlyG to SCWP<sub>BcG9241</sub> was about the same as that observed with SCWP<sub>Ba</sub>. Therefore, both PlyL and, in particular, PlyG bound SCWP<sub>BcG9241</sub>, SCWP<sub>Ba34F2</sub> and SCWP<sub>Ba7702</sub> with high affinity and showed only weak or no affinity to SCWP<sub>Ba14579</sub> and SCWP<sub>Bc10987</sub>. This PlyG binding specificity is consistent with the reported specificity of the  $\gamma$ -phage endolysin for *B. anthracis* strains (see Introduction).

Up to now structural analysis has shown that SCWP<sub>Ba34F2</sub>, SCWP<sub>Ba7702</sub> and SCWP<sub>BcG9241</sub> all contain a  $\rightarrow 4$ - $\beta$ -ManNAc-(1 $\rightarrow$ 4)- $\beta$ -GlcNAc-(1 $\rightarrow$ 6)- $\alpha$ -GlcNAc-(1 $\rightarrow$ ) trisaccharide backbone that is substituted with terminal  $\beta$ -Gal residues on O4 of the  $\alpha$ -GlcNAc residue and by terminal  $\alpha$ -Gal residues on O3 of the  $\alpha$ - and  $\beta$ -GlcNAc residues. The SCWP<sub>BcG9241</sub> structure differs from the *B. anthracis* structures in that 50% of the



**Table I.** Affinities, dissociation rate constants,  $R_{\max}$  and  $\chi^2$  values obtained with SPR for the binding of the C-terminal domain and the full length protein of endolysin PlyL and PlyG to SCWP isolated from different *B. anthracis* and *B. cereus* strains (as indicated); analysis using global fitting

SCWPs	$k_a$ (1/Ms)	$k_d$ (1/s)	$K_D$ (M)	$R_{\max}$ (RU)	$\chi^2$	$k_a$ (1/Ms)	$k_d$ (1/s)	$K_D$ (M)	$R_{\max}$ (RU)	$\chi^2$
	PlyL (C-terminal domain)					PlyL (full-length)				
Ba34F2	<sup>a</sup> $1.42 \times 10^4$	$3.23 \times 10^{-2}$	<sup>a</sup> $1.14 \times 10^{-6}$	334	9.52	<sup>a</sup> $2.12 \times 10^4$	$8.88 \times 10^{-2}$	$7.51 \times 10^{-6}$	132	1.67
	<sup>a</sup> $3.96 \times 10^{-3}$	$1.51 \times 10^{-3}$				<sup>a</sup> $7.14 \times 10^{-3}$	$5.67 \times 10^{-3}$			
Ba7702	<sup>a</sup> $3.98 \times 10^4$	$6.10 \times 10^{-2}$	<sup>a</sup> $2.66 \times 10^{-6}$	252	4.99	<sup>a</sup> $4.71 \times 10^4$	$7.07 \times 10^{-2}$	$2.50 \times 10^{-6}$	239	12
	<sup>a</sup> $7.71 \times 10^{-3}$	$4.98 \times 10^{-3}$				<sup>a</sup> $7.64 \times 10^{-3}$	$5.10 \times 10^{-3}$			
BcG9241	<sup>a</sup> $9.95 \times 10^3$	$6.49 \times 10^{-2}$	<sup>a</sup> $1.68 \times 10^{-5}$	98	0.65	<sup>a</sup> $1.58 \times 10^4$	$3.74 \times 10^{-1}$	$2.63 \times 10^{-5}$	198	1.76
	<sup>a</sup> $4.27 \times 10^{-3}$	$6.76 \times 10^{-3}$				<sup>a</sup> $3.35 \times 10^{-2}$	$3.92 \times 10^{-3}$			
Bc14579	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bc10987	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
	PlyG (C-terminal domain)					PlyG (full length)				
Ba34F2	<sup>b</sup> $3.69 \times 10^3$	<sup>c</sup> $3.01 \times 10^{-3}$	<sup>d</sup> $0.81 \times 10^{-6}$	ND	ND	<sup>b</sup> $5.22 \times 10^3$	<sup>c</sup> $4.25 \times 10^{-3}$	<sup>d</sup> $0.81 \times 10^{-6}$	ND	ND
Ba7702	<sup>b</sup> $6.62 \times 10^3$	<sup>c</sup> $4.16 \times 10^{-3}$	<sup>d</sup> $0.62 \times 10^{-6}$	ND	ND	<sup>b</sup> $3.28 \times 10^3$	<sup>c</sup> $4.60 \times 10^{-3}$	<sup>d</sup> $1.40 \times 10^{-6}$	ND	ND
BcG9241	<sup>b</sup> $1.1 \times 10^4$	<sup>c</sup> $3.82 \times 10^{-3}$	<sup>d</sup> $0.34 \times 10^{-6}$	ND	ND	<sup>b</sup> $1.17 \times 10^3$	<sup>c</sup> $2.9 \times 10^{-3}$	<sup>d</sup> $2.47 \times 10^{-6}$	ND	ND
Bc14579	NB	NB	NB	NB	NB	ND	ND	ND	ND	ND
Bc10987	NB	NB	NB	NB	NB	ND	ND	ND	ND	ND

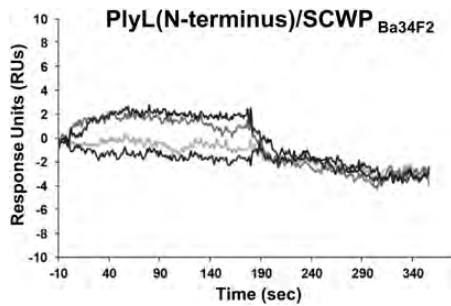
NB, no binding; ND, not determined.

<sup>a</sup>Association and dissociation phase were fitted to a two-state reaction model.

<sup>b</sup>Association phases were calculated from the equation  $k_{\text{obs}} = k_a C + k_d$ .

<sup>c</sup> $k_d$  was calculated using the 1:1 Langmuir dissociation model.

<sup>d</sup> $K_D$  was calculated from  $k_a$  and  $k_d$  values.



**Fig. 5.** BIAcore analysis of the interaction of the endolysin PlyL (N-terminal domain) with SCWP<sub>Ba</sub> samples isolated from *B. anthracis* Sterne 34F<sub>2</sub>. The SCWP concentrations applied were 2000, 1000, 500, 200 and 0 nM (note: the different SCWP concentrations had no effect on the sensorgrams). The sensorgram obtained with the PlyG (N-terminal domain) and SCWP<sub>Ba34F2</sub> showed negative RU at all concentrations and time points (data not shown).

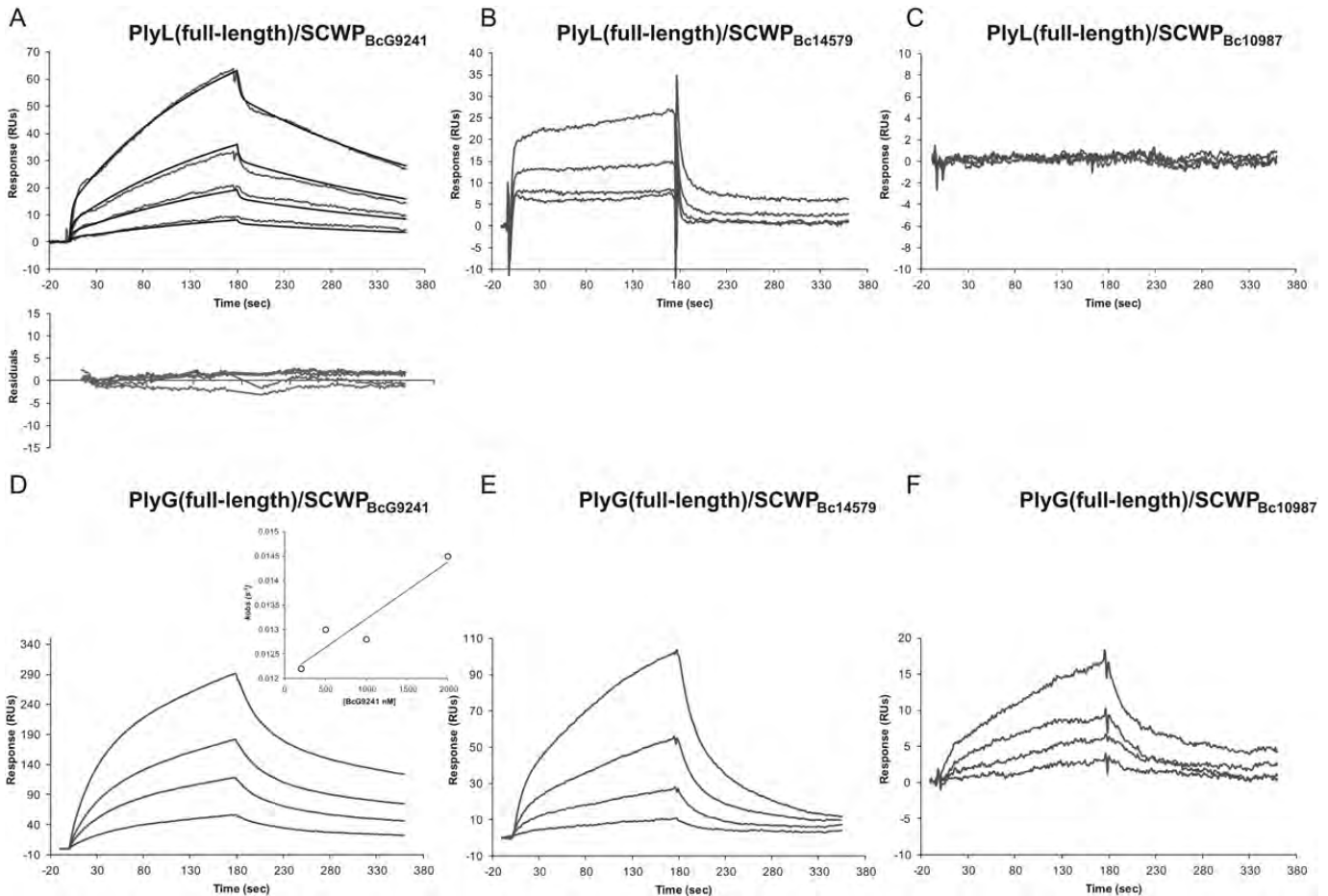
ManNAc residues are additionally substituted by a terminal  $\alpha$ -Gal at O3 (Figure 1). The SCWP<sub>Bc10987</sub> and SCWP<sub>Bc14579</sub> structures contain GalNAc in their trisaccharide aminoglycosyl residue backbone and this backbone is substituted by a single terminal Gal and O-acetyl group (in the case of *B. cereus* ATCC 10987) or by various terminal hexosyl residues including Glc, ManNAc or GlcNAc (as in *B. cereus* ATCC 14579, Candela et al. 2011). Thus, the binding of PlyL and PlyG seems to require a -ManNAc-GlcNAc-GlcNAc- trisaccharide backbone with both GlcNAc residues being substituted with Gal as described. In addition, as the binding affinities of PlyL with SCWP<sub>BcG9241</sub> are weaker than those observed with PlyG, the additional  $\alpha$ -Gal residue attached to the ManNAc in the SCWP<sub>BcG9241</sub> structure seems to lower the PlyL binding affinity but not that of PlyG.

Overall, our results with PlyL and PlyG can be summarized as follows: (i) PlyL and PlyG showed strong affinity to the SCWP<sub>Ba</sub> isolates from strain *B. anthracis* 34F<sub>2</sub>, *B. anthracis* 7702 and *B. cereus* G9241; (ii) the C-terminal domains but not the N-terminal domains of both PlyL and PlyG bound the SCWPs; (iii) both PlyL and PlyG proteins displayed binding specificity toward SCWP<sub>Ba</sub>, i.e. they did not bind SCWP<sub>Bc10987</sub> and only very weakly interacted with SCWP<sub>Bc14579</sub>; (iv) PlyG and PlyL showed comparable binding affinity with both *B. anthracis* SCWP<sub>Ba</sub>; (v) comparison of the affinity constants for the C-terminal domain-SCWP interaction with those for the full-length proteins indicates that both PlyL and PlyG have approximately the same affinity for the endolysin-SCWP ligand complex (Figures 3–7 and Table I).

## Discussion

Bacteriophage endolysins are comprised of two functional domains, a catalytic domain and a domain or ligand conferring substrate recognition (Loessner 2005). To date, only a few ligands of the bacteriophage endolysin have been identified. For the pneumococcal endolysin Cp-1, it has been shown that structural repeats can bind choline, thereby anchoring the protein to choline moieties in the cell wall teichoic acid (WTA; Garcia et al. 1988; Hermoso et al. 2003, 2007). For the endolysin Lyb5 from *Lactobacillus fermentum* bacteriophage  $\phi$ PYB5, it has been shown that it contains LysM domains capable of binding PG (Hu et al. 2010). Recently, it has been shown that the *Listeria* bacteriophage endolysin PlyP35 contains a CBD that recognizes terminal GlcNAc residues in the WTA (Eugster et al. 2011).

The ligands for *B. anthracis* bacteriophage endolysins have not yet been reported. Based on the reported cell-binding properties of the PlyG and PlyL C-terminal domains (Schuch



**Fig. 6.** BIAcore analysis of the interaction between the full-length endolysins PlyL (top panel) and PlyG (bottom panel) and SCWPs isolated from strains *B. cereus* G9241 (A and D), *B. cereus* ATCC14579 (B and E) or *B. cereus* ATCC10987 (C and F). The concentrations of SCWPs used were, from the top to the bottom, 2000, 1000, 500, 200 and 0 nM. The baseline response with no SCWP analyte has been subtracted from the sensorgrams and is not shown. Further details, the experimental conditions were as described in the Figure 3 legend.

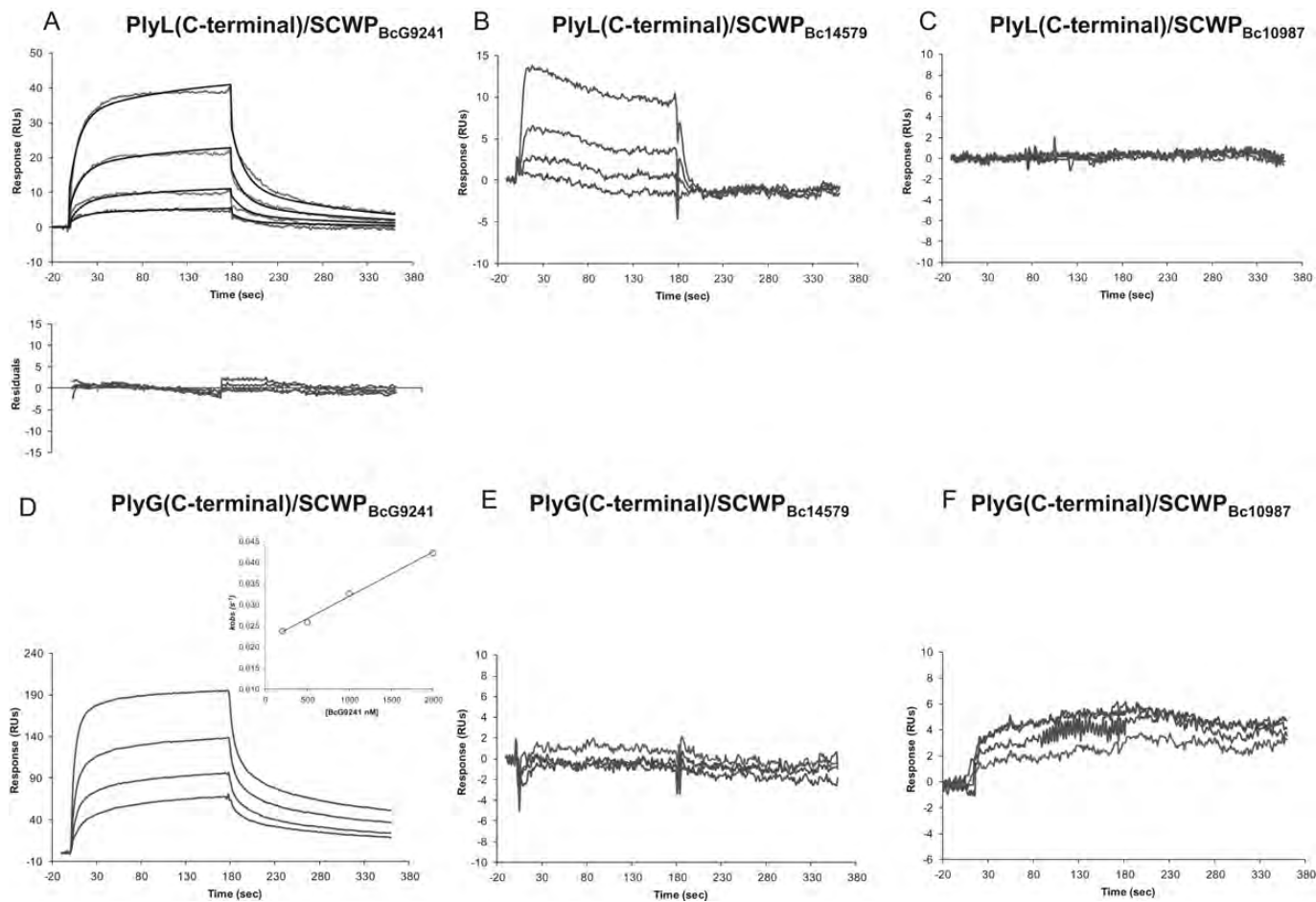
et al. 2002; Low et al. 2005, 2011; Fujinami et al. 2007; Kikkawa et al. 2007, 2008; and observations noted here), we hypothesized that the SCWP<sub>Ba</sub> in the cell walls of *B. anthracis* comprise the ligands for endolysin binding. Using the well-characterized endolysins PlyL and PlyG, and our set of purified and structurally characterized SCWPs isolated from different *B. anthracis* and *B. cereus* strains (Figure 1), we addressed this hypothesis by using SPR analysis to determine whether PlyL and PlyG bind to the different SCWPs. In this paper, we show that the endolysins PlyL and PlyG from *B. anthracis*-specific bacteriophage bind to SCWPs with high affinity and specificity, thus corroborating our hypothesis that the SCWP in the *B. anthracis* cell wall is the ligand for PlyL and PlyG endolysin binding and in this way directs their hydrolytic activity to the PG. Moreover, our results show that the C-terminal domains of PlyL and PlyG, but not their N-terminal catalytic domains, contain the CBD that binds the *B. anthracis* SCWPs.

The affinity constants found for PlyL and PlyG full-length proteins and for the C-terminal domain binding (Table I) are in the range that has been reported for other

protein-carbohydrate interactions (Imberty et al. 2005). They are also in the range that has been found in SPR measurements using small synthetic oligosaccharide analogs modeled after the SCWP<sub>Ba</sub> structure with  $K_D$  values ranging from  $1.1 \times 10^{-6}$  to  $153.5 \times 10^{-6}$  M (Mo et al. 2012). Interestingly, in that study, a synthetic hexasaccharide analog resembling the actual SCWP repeating unit was found to bind with  $K_D$  values of  $23 \times 10^{-6}$  and  $23.3 \times 10^{-6}$  M for the PlyL and PlyG C-terminal domains, respectively. This 10–30-fold weaker binding of the synthetic hexasaccharide when compared with the affinities found for the isolated SCWP<sub>Ba</sub> polysaccharides (each comprised in average of 10 repeating units) could indicate a polyvalent binding mode of action for the isolated SCWP ligands and may explain both (i) why a simple 1:1 binding interaction model did not work for PlyL or PlyG and (ii) why stronger affinities, compared with our SPR measurements, were found for PlyL to whole *B. anthracis* cells (Low et al. 2011).

With the SCWPs established as PlyL and PlyG ligands in the *B. anthracis* cell wall, the question was whether PlyL and PlyG specifically bound *B. anthracis* SCWPs. The inability or





**Fig. 7.** BIAcore analysis of the interaction between the C-terminal domain of endolysins PlyL (top panel) and PlyG (bottom panel) and SCWPs isolated from strains *B. cereus* G9241 (A and D), *B. cereus* ATCC14579 (B and E) or *B. cereus* ATCC10987 (C and F). Concentrations of SCWPs used were same as described in Figure 6 (as described there, the baseline response is also subtracted from the response curves and not shown) and experimental conditions as described in the Figure 3 legend.

only weak binding of SCWP<sub>Bc10987</sub> and SCWP<sub>Bc14579</sub> showed that both endolysins are specific for SCWP<sub>Ba</sub>. The strong Ply binding to SCWPs with a -ManNAc-GlcNAc-GlcNAc- backbone (i.e. the SCWP<sub>Ba34F2</sub>, SCWP<sub>Ba7702</sub> and SCWP<sub>BcG9241</sub>) suggests that this trisaccharide, perhaps together with both GlcNAc residues being substituted with Gal, is the structural motif recognized by the Ply proteins. However, as SCWPs lacking Gal residues have not been investigated yet for binding, the importance for SCWP Gal substitution for Ply binding still needs to be established. As for SCWP<sub>BcG9241</sub>, the partial Gal substitution of the ManNAc residue evidently reduces the affinity of PlyL for this SCWP; in contrast, the binding affinity of PlyG for SCWP<sub>BcG9241</sub> is unaffected (Table I). Despite this difference, for proteins originating from different bacteriophages, PlyL and PlyG have ligand binding specificities with a remarkable overlap in SCWP structural requirements for binding. The underlying molecular reasons for these similar binding requirements are under investigation.

Binding of both PlyL and PlyG with SCWP<sub>Ba</sub> and the sensitivity of *B. anthracis* to the PlyG-producing  $\gamma$ -phage support that SCWP<sub>Ba</sub> is the ligand for these endolysins. Of interest in

this regard is *B. cereus* G9241. In this case, we have shown that PlyG (and PlyL) binds SCWP<sub>BcG9241</sub>, even though it has been shown that strain *B. cereus* G9241 is  $\gamma$ -phage resistant (Hoffmaster et al. 2006). An explanation for this could be that the phage is unable to infect *B. cereus* G9241 cells, e.g. if the cells lack a specific attachment site needed for the phage entry or the polysaccharide capsule of these bacteria (Hoffmaster et al. 2006; Oh et al. 2011) prevent phage binding. Although the  $\gamma$ -phage resistance of *B. cereus* G9241 still awaits an explanation, the  $\gamma$ -phage resistance of these bacteria despite the ability of PlyG to bind SCWP<sub>BcG9241</sub> makes it more likely that  $\gamma$ -phage specificity is not defined by the endolysins' ligand specificity in phage progeny release. Much remains to be learned about SCWPs and lysin action and uses (e.g. whether they have potential for detection and antibacterial uses). Further work is in progress to determine the SCWP structural features required for interaction with endolysins on PlyG lytic activity.

An observation consistent with the identical structures of SCWP<sub>Ba7702</sub> and with SCWP<sub>Ba34F2</sub> was that PlyL and PlyG bound these polysaccharides equally well. Structural analysis

has shown that all the *B. anthracis* SCWP<sub>Ba</sub> structures examined to date have identical ManNAc-GlcNAc-GlcNAc- backbones and attached Gal substitutions (Choudhury et al. 2006). Further, the distal trisaccharide repeat unit is modified by the pyruvylation of the ManNAc residue, 3-O-acetylation of the middle GlcNAc residue and de-N-acetylation of the second GlcNAc residue; the modifications are not present on the remaining SCWP repeat units (Forsberg et al. 2012). These substoichiometric non-carbohydrate modifications, in particular the pyruvyl ketal, are likely requirements of the structural epitopes that are essential for recognition and non-covalent binding to the carbohydrate-binding SLH domain of S-layer proteins and SLH domain-containing proteins (Mesnage et al. 2000; Schäffer and Messner 2005; Kern et al. 2010). However, their role in endolysin binding is unknown. We are currently examining the SCWP from putative mutants affected in these substituents, as well as the chemical modification of the SCWP to determine the role of these non-carbohydrate substituents in both endolysin binding as well and in the binding of SLH domain-containing proteins.

## Experimental

### Bacterial strains used in this study and culture conditions

Strains: *B. anthracis* Sterne 34F<sub>2</sub> (Sterne 1937) and 7702 (Cataldi et al. 1990); *B. cereus* G9241 (Hoffmaster et al. 2004); *B. cereus* ATCC 10987 (Smith 1952) and *B. cereus* ATCC 14579 (Lawrence and Ford 1916). Strains *B. anthracis* 34F<sub>2</sub> and the *B. cereus* strains were obtained from the CDC culture collection. Strain *B. anthracis* 7702 was obtained from T. Koehler, University of Texas/Houston Health Science Center. Strains were grown as described (Leoff, Saile, et al. 2008). Briefly, cells were cultured overnight in brain heart infusion (BHI) medium (BD BBL, Sparks, MD) containing 0.5% glycerol. Washed cells of the overnight culture were used to inoculate four 250-mL volumes of BHI medium in 1-L Erlenmeyer flasks the next morning. Cultures were grown at their optimum growth temperatures, i.e. 37°C for *B. anthracis* and 30°C for *B. cereus* strains, with shaking at 200 rpm. In a mid-log phase, cells were harvested by centrifugation (8000 × g, 4°C, 15 min), washed two times in sterile saline (0.9% w/v NaCl solution), enumerated by dilution plating on BHI agar plates and then autoclaved for 1 h at 121°C before further processing.

### Preparation of bacterial cell walls

The bacterial cell walls were prepared by modification of a previously described procedure (Brown 1973; Leoff, Saile, et al. 2008). The autoclaved bacterial cells (3 × 10<sup>8-9</sup> CFU/mL) were disrupted in 40 mL sterile saline on ice by four 10-min sonication cycles; the cell disruption was checked microscopically. Unbroken cells were removed by a low-speed centrifugation run (8000 × g, 4°C, 15 min). The pellet and supernatant fractions were stored at -70°C. The cell walls were separated from the low-speed supernatants by ultracentrifugation at 100,000 × g, 4°C for 4 h. The resulting cell wall pellets were washed by suspension in cold, deionized water followed by an additional ultracentrifugation at 100,000 × g, 4°C for 4 h and lyophilized.

### Release of phosphate-bound polysaccharides from the cell wall

Phosphate-bound polysaccharides were released from the cell walls by treatment with aqueous hydrogen fluoride (HF; these HF-released SCWPs are, therefore, also in the literature referred to as HF-PSs) in a modified procedure described by Ekwunife et al. (1991). Briefly, the cell walls were subjected to 47% HF under stirring at 4°C for 48 h. The reaction mixture was neutralized with NH<sub>4</sub>OH, subjected to a 10-min low-speed centrifugation (10,000 × g), and the supernatant with the released polysaccharides lyophilized, redissolved in deionized water and subjected to a chromatographic size separation on a BioGel P2 column (Bio-Rad, Hercules, CA, USA). The fractions eluting from the BioGel P2 column were monitored using a refractive index detector. Polysaccharide-containing peaks were pooled, lyophilized and analyzed by gas chromatography-mass spectrometry (GC-MS) as described in the *Glycosyl composition analysis* section. For size approximation, the isolated polysaccharides were chromatographed on a Superose-12 column, using α-1-6 linked dextrans as the calibration standards. Size heterogeneity was observed of the different HF-PSs with molecular masses between 12,000 and 20,000 Da. Hence, for calculations in this study, an average HF-PS/SCWP molecular mass of 16,000 Da was assumed.

### Glycosyl composition analysis

The carbohydrate profiles were determined by GC-MS analysis of the trimethylsilyl methylglycosides as described previously (York et al. 1986). The SCWP fractions were subjected to methanolysis at 80°C for 18 h in methanolic HCl (1 M). The resulting methyl glycosides were *N*-acetylated, trimethylsilylated and then analyzed by GC-MS analysis (5890AGC-MS; Agilent Technologies, Palo Alto, CA) using a 30-m DB-1 fused silica capillary column (J&W Scientific, Folsom, CA). Inositol was used as an internal standard, and retention times were compared with the authentic standards. Composition analysis was done on each sample at least two times.

### Cloning, expression and purification of the endolysins

The PlyL full-length protein and the PlyL C-terminal domain were expressed and purified as described (Low et al. 2005). The PlyL and PlyG N-terminal domains were obtained as explained in Low et al. (2005, 2011). The DNA of the PlyG C-terminal domain was codon optimized (for *E. coli*) and synthesized from amino acids Lys166 to Lys233, based on NCBI accession DQ221100 for the PlyG full-length protein. The resulting construct, with a methionine added in front of Lys166, was inserted into the vector pET15b (Novagen, Darmstadt, Germany) between *Nde*I and *Bam*HI restriction sites. BL21DE3 (Agilent Technologies) competent cells were transformed with pET15b-PlyG-cell wall binding moiety and grown to an OD<sub>600</sub> of 0.8, before being induced with 1 mM isopropyl-β-D-thiogalactoside for 3 h. After induction, the *Escherichia coli* BL21DE3 cells were harvested by centrifugation at 4°C at 6090 × g (Sorvall GS-3 rotor) for 15 min. The lysis buffer (20 mM Tris-Cl, 300 mM NaCl and 0.5% Triton X-100, at pH 7.0) was used to resuspend the cell pellet. Cells were lysed by French-press with three passes at 1000 pound per square inch and clarified by centrifugation at 38,725 × g

(Sorvall SS-34) for 1 h at 4°C. The clarified lysate was loaded directly onto a 5-mL HITRAP Ni-chelating column (GE Healthcare, Piscataway, NJ, USA) equilibrated with 50 mL buffer A (20 mM Tris-Cl and 300 mM NaCl at pH 7.0). Unbound protein was eluted by applying 50 mL buffer A containing 30 mM imidazole. The His-tagged protein was eluted by 0.3 M imidazole in buffer A. A Superdex S200 16/60 (GE Healthcare) gel filtration, equilibrated with 1× phosphate-buffered saline (PBS) (pH 7.4), was then used to further purify the target protein. The molecular weight of the final protein products (i.e. PlyL full length, 26.41 kDa; PlyL C-terminal domain, 11.02 kDa; PlyL N-terminal domain, 20.23 kDa; PlyG full length, 28.43 kDa; PlyG C-terminal domain, 10.1 kDa; PlyG N-terminal domain, 20.21 kDa; all proteins contain a His-tag with the exception of PlyL full length) was confirmed by matrix assisted laser desorption ionization-time of flight mass spectrometry.

#### *Cell-binding assay of the PlyL C-terminal domain to B. anthracis cells*

*Bacillus anthracis* Sterne (34F<sub>2</sub>) cells were grown in 10 mL of luria broth liquid medium to a cell density at OD<sub>600</sub> of 1. The cells were harvested at 19,100 × g (Sorvall table-top centrifuge) for 5 min and resuspended in 10 mL of PBS (pH 7.5; the cell density OD<sub>600</sub> will be ~1). Depending on the 6xHis-tagged PlyL C-terminal domain concentration applied (i.e. ranging from 0, 0.007 to 2.0 μM PlyL C-terminal domain), the protein was prediluted into PBS buffer before adding it to the cells. The total volume of each experiment was 1 mL. The tubes containing the cell-protein mixtures were placed on a rocking platform at room temperature for 10 min. After incubation, the cells were pelleted at 19,100 × g for 5 min using a table-top centrifuge. The pellets were resuspended and washed three times with PBS. Equal volumes of 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer were added to the cells and the final mixture boiled for 1 min at 100°C. Twenty microliters of the samples was loaded onto a 4–20% SDS-PAGE gel (Invitrogen, Grand Island, NY, USA). Equal loading of *B. anthracis* cells was confirmed making use of the intensity of the prominent band of the S-layer proteins Sap and EA1 in Coomassie-stained PAGE gels. For western blots, the penta-His antibody (Qiagen, Valencia, CA, USA) and the Goat horseradish peroxidase conjugated anti-mouse antibody (Calbiochem, Billerica, MA, USA) were used as the primary and the secondary antibodies, respectively.

#### *SPR analysis*

A BIAcore 3000 instrument under the control of BIAcore 3000 software version 3.2 was used for this analysis together with the following materials and solutions: a research grade sensor chip CM5; NHS; *N*-ethyl-*N'*-(3-diethylaminopropyl)-carbodiimide hydrochloride (EDC); 1 M ethanolamine-HCl, pH 8.5; running buffers: either HBS-EP buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 3.4 mM ethylenediaminetetraacetic acid (EDTA) and 0.005% (v/v) surfactant polysorbate P20 at pH 7.4, from BIAcore] or PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4, from EMD); bacteriophage

endolysins PlyL and PlyG full-length, PlyL and PlyG C-terminal and PlyL and PlyG N-terminal domains (prepared as described in the *Cloning, expression and purification* section); SCWPs from strains *B. anthracis* Sterne 34F<sub>2</sub> and 7702, *B. cereus* ATCC 10987, *B. cereus* G9241 and *B. cereus* ATCC 14579 (prepared as described in the section on the release of phosphate-bound polysaccharides from the cell wall); coupling buffer: 10 mM acetate buffer adjusted to appropriate pH values (see below); regeneration buffer (glycine, pH 2.0) were purchased from BIAcore.

The endolysin proteins were immobilized using the standard amine coupling procedures (Löfås and Johnsson 1990) at conditions recommended by the manufacturer: as a preliminary for protein immobilization, the pH scouting was done with coupling buffer (10 mM acetate buffer) adjusted to pH 4.0, 4.5, 5.0 or 5.5. For best protein immobilization, the pH conditions for protein coupling of the different endolysins were chosen as follows: pH 5.5 for the PlyL full length and the N terminus and pH 4.5 for the derived C-terminal protein fragment; and pH 5.0 for PlyG full length and pH 5.5 for the derived C and N terminus. The endolysin was immobilized on the surface of a CM5 sensor chip (BIAcore) using the standard amine coupling chemistry with HBS-EP [10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.005% (v/v) Surfactant P20]. To activate the carboxymethyl dextran surface, a freshly prepared mixture (1:1) of NHS (50 mM in water) and EDC (200 mM in water) was injected for 7 min, then the endolysin (normally 50 μg/mL in coupling buffer; some preparations were coated at a concentration of 100 μg/mL) was injected until the desired level of immobilization (~1000 RU) was achieved (flow rate: 10 μL/min). Any remaining activated carboxylic acid groups were blocked by a 50 μL of 1 M ethanolamine, pH 8.6, followed by a 10-μL injection of 10 mM glycine at pH 2.0 to wash out any unbound endolysin. After immobilization of the ligand, buffer was passed over the surface until baseline stability was obtained. As a reference surface for the refractive index and correction of non-specific binding, a second flow cell was treated in the same manner, but without the addition of endolysin protein. For kinetics studies (adjusted from Xie et al. 2005 and Urbinati et al. 2004), the SCWP analyte samples contained 0, 200, 500, 1000 and 2000 nM of the respective SCWP dissolved in running buffer (10 mM HEPES buffer at pH 7.4 or PBS). The kinetic assays were carried out at 25°C and a flow rate of 10 μL/min. For each analyte concentration, association was measured for 180 s and dissociation was measured for 300 s. After each injection, the surface was regenerated with 30 s injection of 10 mM glycine at pH 2.0, followed by a 30-s injection of 1.5 M NaCl, pH 7.4, in PBS or HEPES buffer at a flow rate of 30 μL/min. The cycle was repeated for every endolysin-SCWP pair.

#### *Data analysis*

All sensorgrams were obtained by a double-referencing procedure as described by Myska (1999) using global fitting. The kinetic data for the different endolysin-SCWP pairs were fitted to different interaction models available in the BIAevaluation software 4.1.1. For the PlyL-SCWP interactions, a two-state binding model resulted in a good fit



between calculated and observed curves. The goodness of fit was determined by the  $\chi^2$  values, as well as the magnitude and distribution of the residual values (defined as the difference between observed and calculated values). In contrast, the PlyG–SCWP interactions did not fit any of the binding models supplied by the BIAevaluation 4.1 software. In the latter case, the association rate constants ( $k_a$ ) were determined from the slope of a plot of the observed association rate constant ( $k_{obs}$ ) as a function of the analyte concentration  $C$ , according to  $k_{obs} = k_a C + k_d$  (inserts in Figures 3, 4, 6 and 7; Yu et al. 2005; Chevigné et al. 2007). The dissociation rate constant ( $k_d$ ) was calculated using the 1:1 Langmuir dissociation model from which dissociation constant,  $K_D$ , was obtained from the ratio of  $k_d/k_a$ .

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### Conflict of interest

None declared.

### Abbreviations

ATCC, American Type Culture Collection; BHI, brain heart infusion; CBD, cell wall-binding domain; EDC, *N*-ethyl-*N'*-(3-diethylaminopropyl)-carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; Gal, galactosyl; GC-MS, gas-liquid chromatography-mass spectrometry; Glc, glucosyl; HBS-EP, 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA and 0.005% [v/v] surfactant polysorbate P20 at pH 7.4; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HF, hydrofluoric acid; HF-PS, HF-released cell wall polysaccharide; HRP, horseradish peroxidase; LB, luria broth; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; PG, peptidoglycan; PlyL, phage lysin L; PlyG, phage lysin G; RU, response unit; SCWP, secondary cell wall polysaccharide; SCWP<sub>Ba</sub>, SCWP from *B. anthracis* strains; SCWP<sub>Ba34F2</sub>, SCWP from strain *B. anthracis* 34F<sub>2</sub>; SCWP<sub>Ba7702</sub>, SCWP from strain *B. anthracis* 7702; SCWP<sub>Bc10987</sub>, SCWP from strain *B. cereus* ATCC 10987; SCWP<sub>BcG9241</sub>, SCWP from strain *B. cereus* G9241; SCWP<sub>Bc14579</sub>, SCWP from strain *B. cereus* ATCC 14579; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel

electrophoresis; SLH, S-layer homology; SPR, surface plasmon resonance; WTA, wall teichoic acid.

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