A structural basis for the allosteric regulation of non-hydrolysing UDP-GlcNAc 2-epimerases

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The non-hydrolysing bacterial UDP-N-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase) catalyses the conversion of UDP-GlcNAc into UDP-N-acetylmannosamine, an intermediate in the biosynthesis of several cell-surface polysaccharides. This enzyme is allosterically regulated by its substrate UDP-GlcNAc. The structure of the ternary complex between the *Bacillus anthracis* UDP-GlcNAc 2-epimerase, its substrate UDP-GlcNAc and the reaction intermediate UDP, showed direct interactions between UDP and its substrate, and between the complex and highly conserved enzyme residues, identifying the allosteric site of the enzyme. The binding of UDP-GlcNAc is associated with conformational changes in the active site of the enzyme. Kinetic data and mutagenesis of the highly conserved UDP-GlcNAc-interacting residues confirm their importance in the substrate binding and catalysis of the enzyme. This constitutes the first example to our knowledge, of an enzymatic allosteric activation by direct interaction between the substrate and the allosteric activator.

Keywords: allosteric; regulation; UDP-GlcNAc; epimerase

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INTRODUCTION

The non-hydrolysing bacterial UDP-GlcNAc 2-epimerases catalyse the reversible conversion of UDP-N-acetylglucosamine (UDP-GlcNAc) into UDP-N-acetylmannosamine (UDP-ManNAc) (Kawamura *et al*, 1978, 1979). The latter is an intermediate in the biosynthesis of bacterial cell-surface polysaccharides and the enterobacterial common antigen (ECA). The ECA is a surface-associated glycolipid common to all members of the *Enterobacteriaceae* family (Kuhn *et al*, 1988). In species such as *Staphylococcus aureus* and *Bacillus anthracis*, the importance of the UDP-GlcNAc 2-epimerase in the biosynthesis of polysaccharides is highlighted by the presence

of two functionally redundant copies of this enzyme (Kiser *et al*, 1999; Read *et al*, 2003). Studies support a mechanism in which the reaction proceeds through an initial anti-elimination of UDP to generate an intermediate 2-acetoamidoglucal, followed by the subsequent *syn*-addition of UDP to yield the product UDP-ManNAc (Tanner, 2002; Fig 1). This bacterial enzyme is related to the bifunctional mammalian UDP-GlcNAc 2-epimerase/ManNAc kinase, a hydrolysing enzyme that converts UDP-GlcNAc into UDP and ManNAc, and phosphorylates the latter into ManNAc 6-phosphate (Hinderlich *et al*, 1997; Stasche *et al*, 1997). The mammalian enzyme catalyses the rate-limiting step in sialic acid biosynthesis and is a crucial regulator of cell-surface sialylation in humans (Keppler *et al*, 1999).

The structure of the UDP-GlcNAc 2-epimerase in *Escherichia coli* shows structural homology to glycosyl transferases, such as T4 phage β -glucosyltransferase, and glycogen phosphorylase (Campbell *et al*, 2000). Although the enzyme was crystallized in the presence of UDP-GlcNAc, only UDP—an intermediate in the reaction—was observed in the structure bound at a site that has been established to be the catalytic site on the basis of mutagenesis studies (Campbell *et al*, 2000; Samuel & Tanner, 2004).

A unique characteristic of the bacterial UDP-GlcNAc 2-epimerases is their allosteric regulation by the substrate UDP-GlcNAc, which acts as an activator. In the absence of this activator, virtually no UDP-ManNAc is epimerized in the reverse reaction (Kawamura *et al*, 1979; Morgan *et al*, 1997; Samuel & Tanner, 2004); however, when trace amounts of UDP-GlcNAc are added, the reaction proceeds towards equilibrium. This indicates that UDP-GlcNAc is required for the enzyme to acquire a catalytically competent conformation. The structural studies of the *E. coli* epimerase discussed above showed that each subunit of the dimer of the enzyme adopted a slightly different conformation owing to a 10° interdomain rotation that was proposed to be a part of the allosteric regulatory mechanism. However, it remained unclear how UDP-GlcNAc could trigger these changes as it was absent from the structure (Campbell *et al*, 2000).

The structure of the non-hydrolysing UDP-GlcNAc 2-epimerase from *B. anthracis* reported here shows a UDP molecule bound to the active site and an adjacently bound UDP-GlcNAc molecule. The UDP and UDP-GlcNAc are hydrogen bonded to each other and with a common arginine residue. This not only identifies the

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Fig 1 | UDP-N-acetylglucosamine 2-epimerase-catalysed reaction. Reactions catalysed by the (A) bacterial and (B) mammalian enzymes.

allosteric site of this enzyme, but also provides the first observation of direct interaction between a substrate molecule and an allosteric activator in an enzyme active site. Residues coordinating the UDP-GlcNAc are highly conserved in non-hydrolysing bacterial UDP-GlcNAc 2-epimerases but not in their hydrolysing mammalian counterparts, providing a target for the development of antibacterial agents.

RESULTS AND DISCUSSION

Overall structure

The structure of the *B. anthracis* UDP-GlcNAc 2-epimerase was solved to 1.7 Å from crystals grown in the presence of UDP-GlcNAc (Table 1). It is very similar to the previously determined structure of the homologous *E. coli* UDP-GlcNAc 2-epimerase in complex with UDP (Fig 2A; Protein Data Bank (PDB) ID 1F6D; Campbell *et al*, 2000) or with UDP-GalNAc (1VGv; Badger *et al*, 2005) and the *Bacillus subtilis* enzyme without substrate (1O6C; Badger *et al*, 2005). Both the latter structures were solved as part of a structural genomics programme and were not characterized functionally. In 1VGv, UDP-GalNAc was seen bound to the same position as the UDP intermediate in both the *B. anthracis* and *E. coli* (1F6D) structures, but it is not a natural substrate of the enzyme, which is probably why the full molecule was captured in the active site. In contrast to the UDP-bound *E. coli* structure (1F6D), both chains of the *B. anthracis* epimerase dimer are in the same conformation. Secondary structure matching superimposition of equivalent C α atoms of the dimer subunits yields an r.m.s.d. of 0.06 Å and 1.88 Å for the *B. anthracis* and *E. coli* (1F6D) enzymes, respectively. Comparison between the closed form of the *E. coli* enzyme (1F6D) and the *B. anthracis* enzyme yields an r.m.s.d. of 1.63 Å for 341 C α atoms. From here onwards, unless otherwise stated, the closed form of the *E. coli* structure (1F6D) will be used for all structural comparisons.

Active site

Clear electron density for both UDP and UDP-GlcNAc molecules was observed in the active site of the *B. anthracis* enzyme (Fig 2B). UDP and 2-acetoamidoglucal are thermodynamically favoured intermediates of the epimerase-catalysed reaction and are released into solution on prolonged incubation of the enzyme with UDP-GlcNAc (Morgan *et al*, 1997; Samuel & Tanner, 2004). All of the interactions between UDP and the epimerase are

conserved between the *B. anthracis* and the *E. coli* enzymes, and a similar presence of water molecules and lack of electron density was observed at the active site of 2-acetoamidoglucal. Although the residues responsible for proton abstraction/addition have not been identified unambiguously, mutations in the *E. coli* enzyme of ionizable residues found in the region where the glucosamine moiety of the substrate would be positioned identified Asp 95 and Glu 131 as candidates for the proton abstraction, and Glu 117 as being involved in the second reaction step. These residues are conserved in *B. anthracis* (as Asp 100, Glu 136 and Glu 122) and are in similar positions in the structure, making them strong candidates for catalytic residues.

This is the first time that UDP-GlcNAc has been observed in the active site of UDP-GlcNAc 2-epimerase. The molecule lies in an extended pocket lined by several hydrophilic side chains and forms hydrogen bonds to the side chains of residues Gln 43, Gln 46, Gln 70, His 44, His 242, Arg 210 and Glu 136 (Fig 3). It also makes hydrogen bonds to the main chain of the enzyme and to water molecules. UDP-GlcNAc makes two hydrogen bonds to the α - and β -phosphates of the adjacent UDP molecule, helping to hold it in place for the second step of the epimerization reaction.

Sequence alignments of UDP-GlcNAc 2-epimerases from several bacterial species show that all of the residues that interact with UDP-GlcNAc through their side chains are highly conserved in the non-hydrolysing enzymes, but are variant in the hydrolysing enzymes (supplementary information online). Therefore, this UDP-GlcNAc-binding site is conserved and probably unique to the non-hydrolysing bacterial UDP-GlcNAc 2-epimerases.

Although the crystals of the *E. coli* enzyme (1F6D) were also grown in the presence of UDP-GlcNAc and belong to the same space group with related unit cell dimensions, they were obtained at pH 5.25 in which the enzyme is only marginally active (Kawamura *et al*, 1979). The *B. anthracis* epimerase crystals were grown in a pH range of 8.2–8.8, which is the optimal pH range for the enzyme. Thus, it might be that UDP-GlcNAc binds optimally to the enzyme only at a higher pH, which might help to explain how we trapped the enzyme in its UDP-GlcNAc-bound form.

Conformational changes induced by UDP-GlcNAc

Comparison between our UDP-GlcNAc 2-epimerase structure and that of the *E. coli* enzyme in complex with UDP (1F6D; Campbell *et al*, 2000) shows that binding of the substrate UDP-GlcNAc

Table 1 | Crystallographic statistics

Data collection	
Wavelength (Å)	1.08090
Resolution range	60–1.7
Number of unique reflections	106,456
Average redundancy	3.5 (3.4)
Completeness	99.4 (99.0)
R _{merge} (%)	8.7 (34.6)
I/σ	14.6 (3.5)
Wilson B-factor (Å ²)	16.1
Refinement	
Number of reflections	
Work set	101,096
Test set	5,360
R _{factor} (%)	22.1
R _{free} (%)	26.2
R.m.s.d. for bond lengths (Å)	0.018
R.m.s.d. for bond angles (deg)	1.76
R.m.s.d. B factor for main chain atoms (Å ²)	1.67
R.m.s.d. B factor for side chain atoms (Å ²)	3.35
Average B-factor (Å ²)	
Main chains	14.8
Side chains	16.7
Waters	27.4
UDP	12.4
UDP-GlcNAc	10.6

Values in parenthesis refer to the highest-resolution shells.
UDP-GlcNAc, UDP-N-acetylglucosamine.

triggers major but localized conformational changes that occur mostly in three loops (His 209–Gly 215, Ile 65–Leu 72 and Val 241–Pro 245; Fig 4) containing the residues that interact with the pyrophosphate and glucosamine moieties of the UDP-GlcNAc. The flipping of the His 209–Gly 215 loop (His 213–Gly 219 in *E. coli*) is the most striking conformational change, and leads to shifts of 6.5 Å and 12 Å in the Cα atom and guanidium group positions of residue Arg 210 (Arg 214). This is in contrast to a shift of only 0.5 Å for the Cα atom of His 209 (His 213), which establishes hydrogen bonds with the β-phosphate of UDP in both the *B. anthracis* and the *E. coli* enzyme structures. This loop flip brings the side chain of Arg 210 into position to make hydrogen bonds to the β-phosphate of UDP, a hydroxyl group of UDP-GlcNAc, the carboxyl group of Glu 136 (Glu 131 in *E. coli*) and a water molecule (Fig 3). Thus, Arg 210 has an important role in anchoring both UDP and UDP-GlcNAc, and, as its guanidium is likely to be fully protonated at the optimum pH of the enzyme (between 7 and 9), it probably stabilizes the leaving UDP in the anti-elimination reaction.

The Ile 65–Leu 72 (Ile 60–Leu 67) loop was partly disordered in the *E. coli* epimerase enzyme structures in which residues 66–69 (61–64) were not seen (Campbell et al, 2000). In the *B. anthracis* structure, it makes four hydrogen bonds from Arg 69 and Gln 70 to the ribose and α-phosphate of UDP-GlcNAc (Fig 3). The conformational changes that occurs on the Val 241–Pro 245 (Val 245–Pro 249) loop are reminiscent of those in the His 209–Gly 215 (His 213–Gly 219) loop, shifting the Cα and Nε2 atoms of residue His 242 (His 246) by approximately 3 Å and 9 Å, respectively, between both structures. These movements bring His 242 into hydrogen bonding position to both the β-phosphate and a hydroxyl of the UDP-GlcNAc molecule.

Importance of UDP-GlcNAc-interacting residues

Bacterial UDP-GlcNAc 2-epimerases are tightly allosterically regulated by the substrate UDP-GlcNAc as, in the absence of this epimer, the rate of epimerization is extremely slow. This and the strict conservation of the UDP-GlcNAc-interacting residues indicate that the conformational changes discussed above are crucial for converting the enzyme to an active state. To investigate this, we generated point mutants with alanine substitutions of UDP-GlcNAc-interacting residues. All mutants, except His44Ala, were soluble and behaved as wild-type enzyme during purification. As the His44Ala mutation completely abolished the solubility of the protein, a histidine to glutamine change was made that overcame the problem of solubility. Kinetic data for the wild-type and mutant enzymes are summarized in Table 2. The K_M for the wild-type enzyme (2.2 mM) is two- to threefold higher than those reported for the *E. coli* (0.6 mM) and *Bacillus cereus* enzymes (1.1 mM), although the k_{cat} is similar to *E. coli* (7.9 versus 7.1 s⁻¹), whereas the Hill coefficient is slightly lower than both (1.4 versus 1.8 s⁻¹; Kawamura et al, 1978, 1979; Morgan et al, 1997; Samuel & Tanner, 2004). A possible explanation is that *B. anthracis* contains two redundant and identical copies of the UDP-GlcNAc 2-epimerase gene in contrast to the single copy present in *E. coli*. Thus, it might still achieve comparable UDP-GlcNAc turnover rates to *E. coli* by using higher amounts of UDP-GlcNAc 2-epimerase.

The Arg210Ala mutant shows a 10-fold increase in K_M and a 400-fold decrease in k_{cat}, indicating that this residue has an important role both in stabilizing the incoming UDP-GlcNAc that enters the active and allosteric sites, and the resulting UDP produced in the anti-elimination reaction. As Arg 210 is hydrogen bonded to a UDP-GlcNAc hydroxyl that interacts with UDP, in addition to its direct hydrogen bond to one β-phosphate of UDP, it essentially has a role in stabilizing the two β-phosphate oxygen atoms of the latter. The lower Hill coefficient of the Arg210Ala mutant indicates that this residue is also directly involved in the allosteric regulation of the enzyme, as the cooperativity between the allosteric and the catalytic sites is significantly reduced, probably through the disruption of interactions in the His 209–Gly 215 loop. Notably, Arg 210 also interacts with Glu 136 (Glu 131) through two hydrogen bonds (one direct and one water-mediated), and at the optimal pH range of the epimerase reaction (pH 7–9), these side chains are likely to be ionized with opposite charges. The mutation Glu131Gln in the *E. coli* epimerase led to a 10,000-fold decrease in k_{cat} (Samuel & Tanner, 2004), and the effect of the disruption of this interaction on Glu136 in the Arg210Ala mutant might explain its large

Fig 2 | Structure of UDP-GlcNAc 2-epimerase. (A) Stereo view of the superimposition of the structures of UDP-GlcNAc 2-epimerase from *Bacillus anthracis* (light blue) and *Escherichia coli* (magenta). UDP (yellow) and UDP-GlcNAc (green) from the *B. anthracis* structure are shown as sticks. (B) $F_o - F_c$ simulated annealing omit electron density map contoured at 3σ for UDP-GlcNAc and UDP calculated as the final model minus these molecules. UDP-GlcNAc, UDP-N-acetylglucosamine.

Fig 3 | Interactions between the enzyme and UDP-GlcNAc. Stereo view of the hydrogen bond interactions between UDP-GlcNAc (pink), UDP (green) and *Bacillus anthracis* epimerase residues (light blue). Water molecules are shown as red spheres and hydrogen bonds as dotted lines. UDP-GlcNAc, UDP-N-acetylglucosamine.

decrease in k_{cat} . Interestingly, Arg210 is conserved only in the non-hydrolysing bacterial UDP-GlcNAc 2-epimerases, whereas Glu 136 (Glu 131) is conserved in all UDP-GlcNAc 2-epimerases, indicating that the crucial role of Arg210 and the conformational changes associated with it are unique to the bacterial non-hydrolysing UDP-GlcNAc 2-epimerases.

The His242Ala mutant also shows a moderate increase in K_M (fivefold) and a pronounced decrease in k_{cat} (36-fold). This residue makes hydrogen bonds to the β -phosphate and to a hydroxyl group of UDP-GlcNAc that interacts with the neighbouring UDP molecule. Thus, it is likely that it has a role in both the binding of UDP-GlcNAc at the allosteric site and the stabilization of the UDP

Fig 4 | Conformational changes triggered by UDP-GlcNAc. The *Bacillus anthracis* UDP-GlcNAc 2-epimerase is shown superimposed with the closed form of the *Escherichia coli* enzyme (Protein Data Bank ID 1F6D; chain B). The largest conformational changes are shown in blue and pink for the *B. anthracis* and *E. coli* enzymes, respectively. The side chains of Arg210 and His242 are shown for both enzymes (asterisks indicate *E. coli*), and UDP-GlcNAc in pink. UDP-GlcNAc, UDP-N-acetylglucosamine.

Table 2 | Kinetic data ($\pm$ s.e.m., $n = 3$) for the epimerization of UDP-GlcNAc by wild-type and mutant *Bacillus anthracis* UDP-GlcNAc 2-epimerase

Enzyme	k_{cat} (s^{-1})	K_{M} (mM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1} \text{mM}^{-1}$)	n
Wild type	7.9 ± 0.3	2.2 ± 0.2	3.6	1.4 ± 0.1
Arg210Ala	0.021 ± 0.001	20.9 ± 2.2	0.001	1.15 ± 0.07
His242Ala	0.22 ± 0.10	10.4 ± 1.0	0.021	1.47 ± 0.15
His44Gln	4.0 ± 0.2	7.3 ± 0.8	0.55	1.21 ± 0.08
Gln70Ala	2.06 ± 0.07	11.1 ± 0.8	0.19	1.24 ± 0.06
Gln43Ala	1.20 ± 0.07	17.2 ± 1.6	0.070	1.77 ± 0.23

UDP-GlcNAc, UDP-N-acetylglucosamine.

intermediate. Interestingly, its unchanged Hill coefficient indicates that the cooperativity is preserved, with minimal effect on communication between the two sites. Although interactions with the UDP-GlcNAc and UDP are disrupted (as shown by the K_{M} and k_{cat}), the conformational regulation remains similar to wild type. The mutants Gln70Ala, Gln43Ala and His44Gln have moderate decreases (two- to sevenfold) in k_{cat} and increases in K_{M} (three- to eightfold). All of these residues form hydrogen bonds with the axial oxygens of the α -phosphate of the UDP-GlcNAc. His 44 also forms hydrogen bonds with one of the axial oxygens of the β -phosphate, and Gln 43 with the carbonyl of the acetyl moiety of UDP-GlcNAc. Thus, it seems that interactions between the epimerase and the α -phosphate of UDP-GlcNAc help in positioning the latter for optimal interaction with the UDP in the catalytic site. The weakened α -phosphate interactions owing to the loss of a few hydrogen bonds in these mutants probably lead to a lower affinity of the allosteric site for UDP-GlcNAc and also as a consequence for the bound UDP. The Hill coefficients for Gln70Ala and His44Gln have also decreased, showing less cooperativity between the catalytic and allosteric site, which is probably due to disruptions of the hydrogen-bonding network in

the Ile 65–Leu 72 loop. Paradoxically, Gln43Ala shows an increase in cooperativity between sites. The reason for this is unclear from the structure, because this mutation would lead to loss of hydrogen bonds to both UDP-GlcNAc and Thr102. Perhaps replacing a larger glutamine residue with a smaller alanine residue reduces the barrier to loop movement, increasing cooperativity when a UDP-GlcNAc binds (although a higher K_{M} means that this happens at higher substrate concentrations). However, it should be noted that the uncertainty range for the measured n value is double that of the others, making confident conclusions difficult. The relatively larger changes in the K_{M} , k_{cat} and Hill coefficient of the Arg210Ala mutant compared with those of the other mutants confirm that this residue is crucial to the conformational changes that lead to the UDP-GlcNAc-induced activation of the *B. anthracis* UDP-GlcNAc 2-epimerase, and to the stabilization of the interaction between UDP-GlcNAc molecules in the active and allosteric site.

Binding of UDP-GlcNAc obstructs the active site
The binding of UDP-GlcNAc to the allosteric site of the enzyme not only triggers closure of the active site but also obstructs its access to the solvent, thus preventing the products of the anti-elimination reaction from diffusing away before they can react again in the *syn*-addition. Consequently, following every catalytic cycle, the active site must open for product release and substrate uptake. These enzymes release the intermediates UDP and 2-acetoamidoglucal into solution at a rate of about 1/400 that of the UDP-GlcAc epimerization (Morgan *et al*, 1997; Tanner, 2002), and, as these are thermodynamically more stable than either the substrate or the product of the reaction, the enzyme must kinetically trap them to avoid their release into solution.

Gene knockout has impaired growth
To examine the significance of the enzyme to *B. anthracis*, a deletion of one of the two copies of the epimerase gene was constructed. The resulting BA5509 mutant was grown alongside the wild-type strain and showed a significant growth defect (Fig 5). We have been unable to obtain a double mutant strain, indicating

Fig 5 | Growth curve of BA5509 mutant and wild-type strains. Each point represents the average of at least three independent experiments. Error bars are not shown, as they are smaller than the symbols used to mark the data points. OD, optical density.

that such a mutant is non-viable; however, even the deletion of a single copy has marked and adverse effects on the growth of the organism. Our findings, taken with the essential nature of UDP-GlcNAc 2-epimerase activity in both *Listeria monocytogenes* (Dubail *et al*, 2006) and *B. subtilis* (Soldo *et al*, 2002), confirm the importance of both the epimerase activity for growth and its viability as an antibiotic target.

In summary, the structure reported indicates that non-hydrolysing bacterial UDP-GlcNAc 2-epimerases use a new allosteric regulatory mechanism that involves direct interaction between one substrate molecule in the active site and another in the allosteric site. This regulatory mechanism helps the enzyme to hold its substrates and stable intermediates in the active site until the completion of a two-step elimination/addition reaction. The conservation of the allosteric site residues in the non-hydrolysing UDP-GlcNAc 2-epimerases indicates that this mechanism is used exclusively by this class of bacterial enzymes, thus providing a selective way of targeting them, particularly in the case of *B. anthracis*, for the development of antibacterial agents.

METHODS

Protein expression and purification. Both UDP-GlcNAc 2-epimerase and UDP-ManNAc dehydrogenase enzymes were expressed and purified as hexahistidine fusion proteins, and purified to homogeneity by affinity, anion and gel filtration chromatography (supplementary information online).

Crystallization and structural determination. Crystals of the *B. anthracis* UDP-GlcNAc 2-epimerase were grown in hanging drops containing an equal volume of protein (5 mg/ml in 50 mM Tris-HCl (pH 8.0), 5 mM UDP-GlcNAc (Sigma, St Louis, MO, USA), 200 mM NaCl and 2 mM dithiothreitol) and well solution (22–26% PEG 4,000–6,000, 100 mM Tris-HCl (pH 8.2–8.8) and 0.2 M Li₂SO₄) and were cryoprotected by mother liquor supplemented with 30% glycerol. Data were collected at the National Synchrotron Light Source (NSLS) beamline X29, and the structure solved by molecular replacement using the structure of the UDP-GlcNAc

2-epimerase without substrate from *B. subtilis* (PDB ID 1O6C) as a search model (Badger *et al*, 2005). The structure was refined with Refmac5 (Murshudov *et al*, 1997), using noncrystallographic symmetry restraints (supplementary information online). The final model contains all 750 protein residues (375 from each chain including 4 vector-encoded residues), 2 UDPs, 2 UDP-GlcNAcs and 577 water molecules. The Ramachandran plot shows 91.6% of residues in the most favoured regions, and 8.1% and 0.3% in the generously and additionally allowed regions, respectively. Figs 2–4 were generated using PyMol (DeLano, 2002).

Enzyme kinetics. The kinetic parameters of the *B. anthracis* UDP-GlcNAc 2-epimerase for the epimerization of UDP-GlcNAc were obtained by a previously described coupled assay with UDP-ManNAc dehydrogenase (Morgan *et al*, 1997; supplementary information online). Initial reaction velocities were fit to the Hill equation using Prism 4 (GraphPad Software Inc., San Diego, CA, USA):

$$v = V_{\max} [S]^n / ([S]^n + K_m^n) \text{ and } k_{\text{cat}} = V_{\max} / [E].$$

Mutant strain construction. The shuttle plasmid pASD2 was used to disrupt gene BA5509 in *B. anthracis* ΔSterne using a previously described method (Bozue *et al*, 2005). Growth of BA5509 and wild-type strains in BHI media was then monitored in a microplate assay at 24 °C (supplementary information online).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>)

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