Biochemical and structural characterization of alanine racemase from Bacillus anthracis (Ames)

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Abstract

Background: Bacillus anthracis is the causative agent of anthrax and a potential bioterrorism threat. Here we report the biochemical and structural characterization of B. anthracis (Ames) alanine racemase (AlrBax), an essential enzyme in prokaryotes and a target for antimicrobial drug development. We also compare the native AlrBax structure to a recently reported structure of the same enzyme obtained through reductive lysine methylation.

Results: B. anthracis has two open reading frames encoding for putative alanine racemases. We show that only one, dal1, is able to complement a D-alanine auxotrophic strain of E. coli. Purified Dal1, which we term AlrBax, is shown to be a dimer in solution by dynamic light scattering and has a Vmax for racemization (L- to D-alanine) of 101 U/mg. The crystal structure of unmodified AlrBax is reported here to 1.95 Å resolution. Despite the overall similarity of the fold to other alanine racemases, AlrBax makes use of a chloride ion to position key active site residues for catalysis, a feature not yet observed for this enzyme in other species. Crystal contacts are more extensive in the methylated structure compared to the unmethylated structure.

Conclusion: The chloride ion in AlrBax is functioning effectively as a carbamylated lysine making it an integral and unique part of this structure. Despite differences in space group and crystal form, the two AlrBax structures are very similar, supporting the case that reductive methylation is a valid rescue strategy for proteins recalcitrant to crystallization, and does not, in this case, result in artifacts in the tertiary structure.

Background

Bacillus anthracis is a soil-dwelling, spore-forming, Gram-positive bacterium that is the causative agent of the zoonotic disease anthrax. Although the disease is most common in wild and domestic mammals, it can also occur in humans when exposed to infected animals or living spores [1]. The severity of anthrax in humans depends on the route of infection. Inhalation of B. anthracis spores can lead to the most severe form of the disease, historically associated with a case-fatality rate as high as 85% [2,3]. The high mortality rate, the existence of a respiratory route of infection and the great resistance of its spores has
made *B. anthracis* the subject of biological warfare research programs in many countries for over 60 years [4]. The United States Centers for Disease Control and Prevention (CDC) has classified anthrax as a category A bioterrorism agent, posing the greatest possible threat to public health and with the ability to spread across large areas [5]. In 2001, the Ames strain of *B. anthracis* was used in a series of bioterrorist attacks that resulted in five fatalities and cost billions of dollars to the US economy [6,7]. As *B. anthracis* spores are resilient, remaining viable and infective for many years, efforts to decontaminate affected facilities are time-consuming and costly. Therefore, it would be of significant importance to public health and security to develop new strategies aimed at containing *B. anthracis* spores upon their release into the environment.

Alanine racemase (EC 5.1.1.1) is an essential enzyme in prokaryotes. The enzyme utilizes a pyridoxal 5′-phosphate (PLP) cofactor to catalyze the racemization of L-alanine to D-alanine, an essential component of the peptidoglycan layer in bacterial cell walls. The lack of alanine racemase function in eukaryotes has made this enzyme an attractive target for antimicrobial drug development [8,9]. The enzyme utilizes a pyridoxal 5′-phosphate (PLP) cofactor to catalyze the racemization of L-alanine, mediated by alanine racemase, inhibits germination (CDC) has classified anthrax as a category A bioterrorism agent, posing the greatest possible threat to public health and with the ability to spread across large areas [5]. In 2001, the Ames strain of *B. anthracis* was used in a series of bioterrorist attacks that resulted in five fatalities and cost billions of dollars to the US economy [6,7]. As *B. anthracis* spores are resilient, remaining viable and infective for many years, efforts to decontaminate affected facilities are time-consuming and costly. Therefore, it would be of significant importance to public health and security to develop new strategies aimed at containing *B. anthracis* spores upon their release into the environment.

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Alanine racemase structures from the human pathogens *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* have been solved [23] and both revealed further insights into the enzyme’s catalytic site that may lead to identification of new, more specific inhibitors. The high-resolution (1.45 Å) structure of alanine racemase (DadX) from *P. aeruginosa* showed evidence for an external aldimine linkage of an unanticipated guest substrate in the active site [23], while the *M. tuberculosis* structure revealed that the narrow entryway to the enzyme’s active site is composed of highly conserved residues distributed in layers beginning at the PLP site [26]. The structure of the DCS-producing *Streptomyces lavendulae* has also been determined [27]. *S. lavendulae* can grow in the presence of DCS, and the structural basis for the slower inhibition rate of DCS on *S. lavendulae* Alr has been attributed to the enzyme’s larger and more rigid active site [27].

Here we report the cloning and characterization of the two genes, *dal1* and *dal2*, from the *B. anthracis* genome with sequence similarities to other bacterial alanine racemase genes. Although expression of *dal2* in a heterologous system failed, we have successfully expressed and purified the gene product of *dal1*, which we term Alr, and performed its kinetic and structural characterization.

Recently another group has reported that *B. anthracis* alanine racemase crystallization required reductive methylation [28]. Interestingly we have not found this to be the case. However, the availability of both structures, one with and one without methylation, allows for a careful comparison to be performed. Reductive methylation has been employed previously to obtain atomic structures for proteins recalcitrant to crystallization [29-33]. Due to its reported successes, this method is becoming more utilized [28,34,35]. Nevertheless, there may be concerns as to how methylation impacts protein structure. Our analyses of both structures suggest that despite differences in space group and crystal lattice, reductive methylation does not significantly alter the structure of alanine racemase from *B. anthracis*.

**Results and Discussion**

**Sequence analysis of the Bacillus Dal proteins**

The sequences for both *dal1* and *dal2* genes amplified in our laboratory from *B. anthracis* (Ames) genomic DNA are 100% identical to those previously deposited in GenBank (*dal1* GenElD: 1087014 and *dal2* GenElD: 30262102) [36]. The protein sequences encoded by *dal1*
and dal2 both contain the characteristic motifs expected for members of the alanine racemase family, such as a PLP-binding site near the N-terminus, the two key conserved catalytic amino acid residues Lys41 (Alr\textsubscript{Mtb} numbering) and Tyr270, and a set of conserved residues making up the entrance corridor to the alanine racemase active site [26] (Figure 1). The gene product of dal1, which we term Alr\textsubscript{Mtb}, is identical to the alanine racemase protein previously associated with germination in \textit{B. anthracis} spores [37] and shares 57% amino acid identity with Alr\textsubscript{Gst}. Dal2, on the other hand, shows 41% sequence identity to Alr\textsubscript{Gst} and 40% identity to Alr\textsubscript{Gst}.

\textbf{Complementation analysis}

In order to confirm that both dal1 and dal2 genes encode functional alanine racemases we expressed these genes in a D-alanine auxotrophic strain of \textit{E. coli}, MB2795 [38]. Expression of the dal1 gene from \textit{B. anthracis} or dal2 from \textit{P. aeruginosa} fully restored the wild-type phenotype. Cells transformed with pET28-TEV failed to grow, as did those transformed with the \textit{B. anthracis} dal2 gene (data not shown).

\textbf{Overexpression, purification and biochemical characterization of Dal proteins}

We used strain BL21(DE3), pLysS of \textit{E. coli} to express dal1 and dal2 recombinant gene products. While dal1 was expressed successfully, the expression of dal2 failed repeatedly, even when conditions such as temperature, IPTG concentration, or strain background were changed (data not shown). Sequencing of the plasmid construct revealed no obvious errors, and our expression system has been successfully used for numerous proteins in the past. We have no conclusive explanation for our inability to express dal2 at measurable levels in \textit{E. coli}. While the \textit{orf} appears to encode an alanine racemase enzyme, it clearly is not expressed in the T7 overexpression system, possibly also explaining the lack of complementation observed in

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{alignment.png}
\caption{Structure-based alignment of alanine racemases from \textit{B. anthracis} (Alr\textsubscript{Bax}), \textit{G. stearothermophilus} (Alr\textsubscript{Gst}), \textit{M. tuberculosis} (Alr\textsubscript{Mtb}), \textit{S. lavendulae} (Alr\textsubscript{Sla}) and \textit{P. aeruginosa} (DadXPao). The initial alignment was performed using EXPRESSO (3DCoffee) [63] and adjusted manually upon inspection of the superimposed structures. An asterisk marks the location of the Lys residue bound to PLP, the diamond marks the location of the Tyr residue that functions as the second base in the racemase reaction, a bullet denotes the location of the carbamylated lysine found in other alanine racemase structures and replaced by a chloride ion on Alr\textsubscript{Mtb}. I and M denote residues found in the middle or the inner layer of the active site entry way along with their position in the entryway.}
\end{figure}
our earlier experiments. Given that, to our knowledge, there are no reports characterizing dal2 in the literature, we are led to believe that this gene is not usually expressed in its homologous host. As overproduction of Dal2\textsubscript{Bax} failed, all subsequent work was performed with dal1 to yield a product, which we term Alr\textsubscript{Bax}. Alr\textsubscript{Bax} was purified to homogeneity and displayed a single peak on molecular sieve chromatography.

Previous studies have suggested Alr\textsubscript{Bax} might exist partly as a tetramer in solution [34]. We have used dynamic light scattering (DLS) to determine that Alr\textsubscript{Bax} has a hydrodynamic radius of 3.7 nm, corresponding to a molecular weight of 93 kDa. As the calculated molecular weight of Alr\textsubscript{Bax} is 43.7 kDa, this enzyme is unambiguously a dimer (ca. molecular weight 87.4 kDa) in solution under the conditions of this experiment. These measurements were made on a monodisperse solution of Alr\textsubscript{Bax} in which 99.9% of the mass was accounted for by the single peak at 3.7 nm.

We find that purified Alr\textsubscript{Bax} has a $K_{\text{m}}$ for D-alanine of 2.8 mM and a $V_{\text{max}}$ of 101 U mg$^{-1}$, where one unit was defined as the amount of enzyme that catalyzed racemization of 1 μmol of substrate per minute. These kinetic parameters for racemization of L- to D-alanine of Alr\textsubscript{Bax} fall in the range of what has been observed before for other bacterial alanine racemases [38-40]. Interestingly, despite the high identity levels observed for residues in the active site of Alr\textsubscript{Bax} and Alr\textsubscript{Gst} (Figure 1), the $V_{\text{max}}$ for the anthrax enzyme is one order of magnitude lower than the one reported for the G. stearothermophilus enzyme and closer to that observed for alanine racemases isolated from other pathogenic organisms. Our kinetic characterization reinforces previous observations that there is a very wide dynamic range in kinetic constants for alanine racemase, despite the sequence and structural similarities of their active sites.

**Description of the Overall Structure of Alr\textsubscript{Bax} from B. anthracis**

Consistent with other alanine racemases, the tertiary structure of Alr\textsubscript{Bax} is a homodimer formed by head-to-tail-association of two monomers (Figure 2). Each monomer is crystallographically distinct in this crystal form (Table 1), but the two monomers have very similar folds. The rms difference obtained for their C$_\text{α}$ atoms after least-squares superposition is 0.22 Å. Alr\textsubscript{Bax} monomers consist of two structurally distinct domains. Residues in the N-terminal (16–245) fold into an eight-stranded α/β barrel, while the C-terminal residues (246–389) and the first 15 N-terminal amino acids are part of a predominantly β-structure. The homodimer displays two active sites, formed by residues from the N-terminus of one monomer and residues from the C-terminus of the other monomer. The PLP cofactor forms a covalent bond to Lys41 and points at the center of the α/β barrel. As previously observed for Alr\textsubscript{Gst} [22], extra density was present in the active of Alr\textsubscript{Bax}, which we model here as a molecule of acetate.

**Structural comparisons of Alr\textsubscript{Bax} with closely related enzymes**

Below we compare Alr\textsubscript{Bax} to the highly active Alr from the non-pathogenic bacterium G. stearothermophilus (Alr\textsubscript{Gst}) as well as the less active Alrs from pathogenic bacteria P. aeruginosa (DadX\textsubscript{Pao}) and M. tuberculosis (Alr\textsubscript{Mtb}). We also compare Alr\textsubscript{Bax} to the Alr from the DCS-producing bacteria S. lavendulae (Alr\textsubscript{Sla}). These enzymes share between 26 and 57% sequence identity (Figure 1 and Table 2). The crystal structure of native Alr\textsubscript{Bax} reveals some structural features that may be responsible for its slower catalytic rate and suggests regions that might be targeted in designing inhibitors of this enzyme.

As noted, the B. anthracis Alr\textsubscript{Bax} secondary structure and general fold closely resembles that seen for other alanine racemases [23]. However, there are a few small topological differences between the structures of Alr\textsubscript{Bax} and Alr\textsubscript{Gst}. Alr\textsubscript{Bax} is five residues longer than Alr\textsubscript{Gst} three of the five extra residues in Alr\textsubscript{Bax} extend Helix 8 by one turn; while the remaining two extra residues locate to the very N-terminus of Alr\textsubscript{Bax}. Helix 8 does not take part in the enzyme’s active site nor does it make intermonomer contacts, therefore, we do not anticipate this secondary structure to play a critical role in Alr\textsubscript{Bax} function.

Least-squares superposition of C$_\text{α}$ atoms from N- and C-terminal domains from Alr\textsubscript{Gst}, Alr\textsubscript{Sla}, Alr\textsubscript{Alb} and DadX\textsubscript{Pao} to the equivalent domains in Alr\textsubscript{Bax} reveals average rms differences ranging from 1.10 to 2.30 Å. The rms differences correlate with sequence identity levels (Table 2). Superposition of the N-terminal domains of Alr\textsubscript{Bax} and Alr\textsubscript{Gst} reveals significant C$_\text{α}$ deviations (≥ 1.8 Å) for residues in three loops (residues 121–125, between H6 and S6; residues 198–202, between H8 and S8; residues 215–219, between H9 and S9) and residues 148–158 on H7. These regions all locate to the protein surface and have no reported role in homodimer formation or substrate binding and catalysis. On the other hand, superposition of the C-terminal domains of Alr\textsubscript{Bax} and Alr\textsubscript{Gst} shows no regions with C$_\text{α}$ rms differences greater than 1.4 Å.

**Alr\textsubscript{Bax} and Alr\textsubscript{Gst} have a similar hinge angle between N- and C-terminal domains**

The overall rms differences among various bacterial alanine racemases (Table 2) suggest that despite their topological similarity there are notable structural differences between their individual domains. It has been reported previously that the hinge angle between N- and C-terminal domains varies among different alanine racemases [23]. It is due to this difference that monomers from dif-
Table 1: Data-collection and structure-refinement statistics.

| Data collection                  |                  |
|----------------------------------|------------------|
| Space group                      | P2_1             |
| Unit-cell parameters             | a = 49.62 Å, b = 141.27 Å, c = 60.12 Å |
|                                  | α = γ = 90.00°, β = 103.11° |
| Observations                     | 150,355 (12,217) |
| Unique reflections               | 53,396 (5,695)   |
| Resolution (Å)                   | 32.79–1.95 (2.06–1.95) |
| Completeness (%)                 | 91.3 (67.1)      |
| R_{merge} (%)                    | 2.9 (15.6)       |
| Mean (I/σ(I))                    | 22.2 (5.4)       |
| Redundancy                       | 2.8 (2.1)        |

Refinement statistics

| Resolution (Å)                   | 32.79–1.95 (2.01–1.95) |
| Reflections                      | 51,760 (2,518)        |
| Total atoms                      | 6,436                |
| R_{work} (%)                     | 16.0 (19.40)         |
| R_{free} (%) (for 1627 reflections) | 20.1 (23.4)        |
| Average B factor (Å²)            |                      |
| main chain                       | 34.7                 |
| side chain                       | 36.6                 |
| PLP                              | 29.7                 |
| RMS deviations                   |                      |
| bond length (Å)                  | 0.017                |
| bond angles (deg.)               | 1.46                 |
| no. of residues                  | 772                  |
| no. of protein atoms             | 6038                 |
| no. of PLP atoms                 | 30                   |
| no. of acetate atoms             | 8                    |
| no. of water atoms               | 358                  |
| no. of chloride ions             | 2                    |

Values in parentheses are for the highest resolution shell.
Table 2: Average r.m.s.d. (Å) between the Cα atoms of AlrBax and other Alrs

| Alanine Racemase | Whole monomers | N-terminal domains | C-terminal domains | Active site |
|------------------|----------------|-------------------|-------------------|-------------|
| AlrGal            | 1.10 (57%)     | 1.07 (57%)        | 0.60 (59%)        | 0.45 (73%)  |
| AlrStl            | 1.71 (37%)     | 1.66 (36%)        | 1.12 (39%)        | 0.73 (51%)  |
| AlrMsb            | 1.84 (39%)     | 1.64 (36%)        | 1.11 (40%)        | 0.83 (44%)  |
| DadXPan           | 2.30 (26%)     | 1.82 (26%)        | 1.43 (32%)        | 0.81 (41%)  |

Numbers in parenthesis denote sequence identity with AlrBax. aCalculated using monomer A. bCalculated using residues 4–245. cCalculated using residues 246–389. dCalculated using residues 39–45, 63–67, 84–88, 103–107, 127–140, 163–171, 203–210, 221–228, 356–363 from monomer A and 268–271 and 314–319 from monomer B.
If the position of residues taking part in intermonomer contacts is conserved among various Alrs, we would expect the colored spheres to form tight clusters, containing superimposed red, green, blue, yellow and pink spheres. Indeed, as shown in Figure 5, most of the intermonomer contacts from various Alrs are found in clusters and thus are conserved among various Alrs. It is important to keep in mind that the number of residues taking part in intermonomer contacts varies among the analyzed Alrs. For AlrBax, 94 of its 389 residues take part in intermonomer contacts and both N- and C-terminal domains contribute an almost equal number (44 and 50, respectively) of residues to the interface. The total number of residues in the interface of AlrGst, AlrSla, AlrSan and DadXPao is slightly smaller than in AlrBax. Nevertheless, for all analyzed structures, both domains contribute almost equally to the monomer-monomer interface.

At its dimer interface, the AlrBax structure displays a larger surface area and higher number of polar interactions than AlrGst, AlrSla and DadXPao (Table 3). Not surprisingly, most of the additional buried surface area observed for AlrBax results from the interactions involving N- and C-terminal residues described in the hinge angle analysis above. If residues from the N-terminus (4–10) and C-terminus (383–389) of AlrBax are excluded from the calculation, the intermonomer surface area of AlrBax is reduced from 3,500 to 2,500 Å², making it similar to the values found for AlrSla and DadXPao (~2700 Å²) (Table 3).

**AlrBax PLP-binding and active site**

As observed for other Alrs, the active site of AlrBax is formed by residues from both monomers, with the two catalytic bases Lys41 and Tyr270’ found in different monomers. In the AlrBax structure, Lys41 is seen covalently linked to the PLP cofactor. As was observed for one of the AlrGst structures (1sft) [22], we have identified extra density in the active site of AlrBax which we have modeled as a molecule of acetate. Acetate, which was present in our crystallization solution, is an inhibitor of Alr [22] and its carboxylate group is thought to bind the enzyme active site in the same way the carboxylate group from alanine is expected to do [22]. The oxygen atoms from the acetate molecule in our model are within hydrogen bonding distance to the side chain oxygen from Tyr289’, the main chain nitrogen from Met317’ and, perhaps more importantly, to the side chain nitrogen atom from the catalytic Lys41 residue (Figure 6).

The identity and position of active site residues is strongly conserved among various Alrs (Table 2). As a result, the hydrogen bonding network found for the PLP molecule in the active site of AlrBax is similar to the one observed for other Alrs. In AlrBax, side chain atoms from Tyr45, Arg138, Arg24, His168, Ser209 and Tyr359 establish hydrogen bonds to atoms in the PLP cofactor (Figure 6). These resi-
dues are strictly conserved for Alr\textsubscript{Gst}, Alr\textsubscript{Mtb}, Alr\textsubscript{Sla} and DadX\textsubscript{Pao}, and have similar orientations in the PLP-binding site of their respective enzymes. The PLP in Alr\textsubscript{Bax} also hydrogen bonds with main chain atoms from Ser\textsubscript{209}, Gly\textsubscript{226} and Ile\textsubscript{227}. The first two of these residues is strictly conserved in Alr\textsubscript{Gst}, Alr\textsubscript{Mtb}, Alr\textsubscript{Sla} and DadX\textsubscript{Pao}. The third would be as well but in Alr\textsubscript{Sla}, the Ile\textsubscript{227} is replaced by a leucine residue. Perhaps a more significant difference is the presence in Alr\textsubscript{Sla} and Alr\textsubscript{Mtb} of a tryptophan residue in place of Alr\textsubscript{Bax} Leu\textsubscript{87}. A tryptophan residue at this position is one of the differences found between the active sites of the slower enzymes from \textit{M. tuberculosis} and \textit{S. lavendulae} and the faster Alr from \textit{G. stearothermophilus}. In Alr\textsubscript{Sla} and Alr\textsubscript{Mtb}, the Ne atom of this tryptophan makes a water-mediated hydrogen bond to O\textsubscript{3} from PLP. Although this extra interaction may have a role in catalysis it does not seem to reduce the size of the Alr\textsubscript{Sla} and Alr\textsubscript{Mtb} active sites as the loop that harbors this tryptophan residue is shifted away (\(\sim\)2.1 Å) from the PLP cofactor when compared to the same loop in Alr\textsubscript{Bax}. Mutagenesis studies could thus be performed in order to evaluate the impact of this tryptophan residue for enzyme catalysis.

One striking difference in the active site involves Asn\textsubscript{131}, which in other alanine racemases is generally a carbamylated lysine that participates in a hydrogen bond with the residue homologous to Arg\textsubscript{138}. In Alr\textsubscript{Bax} however, we note a prominent chloride ion that is located near Arg\textsubscript{138} in the active site (Figure 6). This chloride ion has not been described in Alr structures from other species and it was originally modeled by us as a water molecule. However, the resulting low B-factor (\(\sim\)10 Å\(^2\)) and its hexa-coordination with three water molecules and atoms N\textsubscript{δ2} from Asn\textsubscript{131} and Ne and Nη\textsubscript{2} from Arg\textsubscript{138} suggested the presence of a chloride ion. Notably, there is no chloride present in the crystallization buffer and we can only assume that the enzyme binds so tightly to this halide that it is carried over from the enzyme's purification. The chloride ion is also observed on the Alr\textsubscript{Bax} structure obtained following lysine reductive methylation [30]. The presence of a chloride ion in two independent structures reinforces
the idea that this ion plays an important structural role in Alr\textsubscript{Bax}. Other Alrs have a negative charge at the same position, but the charge has always been from a carbamylated lysine residue (Figure 6). In the Alr\textsubscript{Mtb} structure a carbamylated lysine was not noted but the side chain density for this lysine was poor. Like the chloride ion in Alr\textsubscript{Bax}, the carbamyl group found in other Alrs hydrogen bonds with Ne and N\textsubscript{2} from the active site arginine (Arg138 in Alr\textsubscript{Bax}), thus positioning this residue in the active site. The general conservation of the modified lysine residue among various Alrs and its role in positioning the active site arginine indicates that the presence of a negative charge at this position is critical for enzyme catalysis. As Alr\textsubscript{Bax} lacks the conserved lysine residue necessary for carbamylation it has apparently drafted a chloride ion to fill the same role for this species. It is open to speculation whether the addition of chloride chelators like SPQ (6-methoxy-N-(3-sulfopropyl)-quinolinium) would affect the enzyme activity and whether it might be possible to design specific inhibitors for Alr\textsubscript{Bax} based on this unique interaction.

In Alr\textsubscript{Bax} in addition to the interactions facilitated by the chloride ion, Arg138 is further positioned by the side chain oxygen of Thr316'. Further, an alignment of 105 Alrs, having between 24% to 99% sequence identity to Alr\textsubscript{Bax}, revealed that the presence of an asparagine at the equivalent position to Asn131 in Alr\textsubscript{Bax} is always accompanied by the presence of a threonine residue equivalent to Thr316' (data not shown) suggesting that this interaction with Arg138 would be a conserved feature of alanine racemases with active site structural chlorides. Sequences of Alrs that contain a lysine in position 131 almost always have an accompanying serine or a cysteine residue in position equivalent to Alr\textsubscript{Bax} Thr316'. In the case of Alr\textsubscript{Pao} this serine is involved in an equivalent active site arginine interaction. The exception to this latter observation is Alr\textsubscript{Sla} which has an alanine at this position. It is important to note that there is not really a specific chloride-binding motif as the residues that interact with Cl\textsuperscript{-} in Alr\textsubscript{Bax} are the same that interact with the carbamylated lysine in the other structures.

**The active site entryway of Alr\textsubscript{Bax}**

Residues from loops in the \(\alpha/\beta\) barrel domain of one monomer and residues from the C-terminal domain of the second monomer make up an entryway to the active site and the PLP binding site. The active site entryway of Alr has been previously divided in inner, middle and outer layers, starting from the PLP binding pocket and moving towards the protein surface [26]. Residues in the inner and middle layers show strong conservation among various Alrs [26]. For Alr\textsubscript{Bax} residues Tyr270', Tyr359, Tyr289' and Ala172 constitute the inner layer, while residues Arg314', Ile357, Arg295' and Asp173 make up the middle layer. These residues are absolutely conserved between Alr\textsubscript{Bax} and Alr\textsubscript{Gst}, Alr\textsubscript{Mtb}, Alr\textsubscript{Sla} and DadX\textsubscript{Pao}. The outer layer for the active site entryway of various Alrs displays less conservation, but in this region Alr\textsubscript{Bax} contains...
Table 3: Intermonomer interactions for alanine racemases

| Alanine racemase | Intermonomer surface area (Å²) | Hydrogen bonds | Salt bridges |
|------------------|-------------------------------|----------------|--------------|
| Alr\textsubscript{Bax} | 3536                          | 38             | 9            |
| Alr\textsubscript{Gst}    | 3083                          | 31             | 12           |
| Alr\textsubscript{Sla}    | 2798                          | 20             | 7            |
| DadX\textsubscript{Pao}   | 2788                          | 14             | 7            |

An Asn271' while Alr\textsubscript{Gst}, Alr\textsubscript{Mtb}, Alr\textsubscript{Sla} and DadX\textsubscript{Pao} contain a glycine. As a result of this substitution, the entryway is somewhat more restricted than the ones observed for other alanine racemases. Finally, for Alr\textsubscript{Bax}, a conserved pair of acidic residues (Asp-Glu) is found at positions 173 and 174, which are located in the middle and outer layers of the entryway. Identical residues are found in the same position for Alr\textsubscript{Gst}, Alr\textsubscript{Sla} and DadX\textsubscript{Pao}, but for Alr\textsubscript{Mtb}, a much slower alanine racemase, these two residues are (Asp-Lys). This site has recently been shown to be important catalytically, as making this Asp-Glu to Asp-Lys change at the same position in E. coli alanine racemase has been shown to significantly decrease its catalytic rate [45].

Structural comparison of native and reductively methylated alanine racemases from B. anthracis

Recently, the structure of Alr\textsubscript{Bax} after reductive methylation of its lysine residues (Alr\textsubscript{BaxRM}) has been reported [28]. In that report, the unmodified protein failed to crystallize. Scientists at the Oxford Protein Production Facility (OPPF) and the York Structural Biology laboratory reported that extensive crystallization trials (approximately 800 conditions) with native Alr\textsubscript{Bax} proved unsuc-

Figure 6
Organization of the active site residues in B. anthracis Alr is facilitated by a chloride ion. (A) Electron density map (contoured at 1.5σ in the final refined 2Fo-Fc map) showing details of the active site for Alr\textsubscript{Bax}. (B) Structural alignment of residues making the active site of various Alrs (TB structure was not included). For all available Alr structures, Arg138 makes polar contacts to the PLP and, possibly, to the substrate. In Alr\textsubscript{Bax}, this arginine residue is positioned in the active site by a chloride ion (Cl\textsuperscript{-}). Polar contacts between the chloride ion and Asn-131 and Arg-138 are shown in Panel A by dashes. For all other alanine racemase structures available to date the equivalent interactions are mediated by a carbamylated lysine (shown in Panel B). Residues in the active site of various Alrs are shown as a stick model. In Panel A, the acetate molecule and the modified lysine residue (LLP) are depicted as ball and stick models; carbons are colored in green, nitrogen in blue, oxygen in red, phosphate in orange, sulfur in yellow and the chloride ion is depicted as a light green sphere. In Panel B residues are shown as stick model and are colored according to the legend on figure 3; the PLP cofactors are shown as ball and stick models. In both panels, primed numbers denote residues from the second monomer.
cessful and that reductive lysine methylation was essential for crystallization of the protein [34,46]. Based on data from mass spectroscopy and on the methylated crystal structure of AlrBax, Au and colleagues concluded that the N terminus and 18 out of the 20 lysines in AlrBax were methylated after the protein was treated with dimethyl-amine borane complex and formaldehyde.

Reductive methylation modifies all free primary amines in a protein molecule (NH groups from lysine residues and the N terminus) to tertiary amines. This modification of lysine residues, especially those found on the protein surface, offers an opportunity to change a protein’s crystallization properties and is a proven method to rescue proteins recalcitrant to crystallization [28-33,35,47]. However, there are few structural studies showing that reductive alkylation does not alter a protein’s structure, especially of proteins that do not readily crystallize. One study [48] reporting on the effects of reductive lysine methylation on HEW lysozyme found that crystals were formed under different conditions and with a different crystalline lattice than observed for the unmodified enzyme. Nevertheless, the structures of both modified and unmodified enzymes showed no significant structural differences and their superimposed Ca atoms had an rms difference of only 0.4 Å [48]. The availability of native and modified structures for AlrBax, therefore, offers another opportunity to evaluate the impact of reductive lysine methylation, this time on a protein more recalcitrant to crystallization.

In our hands, AlrBax protein readily formed small crystals using commercially available crystallization screens. Notably our form contains eight additional residues at the C-terminus that remain following cleavage of a C-terminal His-tag using TEV protease. These residues are not involved in crystal contacts, but still could have an influence on crystallization. Our initial crystallization conditions required extensive fine-tuning, and the addition of the glutathione additive proved important for obtaining diffracting quality crystals. Moreover, finding the proper conditions for freezing AlrBax crystals without compromising diffraction quality proved challenging. For simplicity’s sake we have referred to this form of AlrBax as unmethylated or native. Our review of the expression and purification protocols for both native and alkylated enzymes suggests that they were very similar. Also, modified and unmodified AlrBax crystallize under similar conditions, despite a reported small reduction in the isoelectric point of the modified enzyme (1529.7 Å² vs. 918.4 Å², respectively). Further, these contacts are often mediated by methylated lysine residues found at the protein surface (Figure 7). In monomer A from AlrBaxRM, 6 out of the 18 modified lysines contact protein atoms from both monomers in adjacent asymmetric units. For monomer B, 9 modified lysines engage in crystal contacts; contacting

Interestingly, the modified enzyme was crystallized at 60 mg/ml while the native structure was obtained from crystals grown at 15 mg/ml.

Despite similar crystallization conditions native and modified AlrBax crystals show different crystalline lattices and solvent content. Native AlrBax crystals are monoclinic with space group P2₁ and unit cell parameters a = 49.6 Å, b = 141.3 Å, c = 60.1 Å and β = 103.11°. On the other hand, crystals for the methylated enzyme are orthorhombic in space group P2₁2₁2₁ with cell dimensions of a = 57.6 Å b = 88.4 Å and c = 139.0 Å. Crystals for the modified enzyme display a lower solvent content (38% vs. 48%) and a higher packing density (1.99 Å³/Da vs. 2.35 Å³/Da) than native crystals.

**Crystal contacts comparison**

The total surface area found in crystal contacts for the reductively methylated enzyme is 1.7 times larger than that found for the native enzyme (1529.7 Å² vs. 918.4 Å², respectively). Further, these contacts are often mediated by methylated lysine residues found at the protein surface (Figure 7). In monomer A from AlrBaxRM, 6 out of the 18 modified lysines contact protein atoms from both monomers in adjacent asymmetric units. For monomer B, 9 modified lysines engage in crystal contacts; contacting

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**Figure 7**

**Difference in crystal contacts following reductive methylation of secondary amines on AlrBax.** The position of Ca atoms from residues making crystal contacts are shown as colored spheres superimposed onto the ribbon diagram of AlrBax (shown in green). Methylated lysines involved in crystal contacts are shown in red; other residues involved in crystal contacts for the methylated structure only are shown in yellow. Residues implicated with crystal contacts for the native structure only are shown in blue. Residues found to make crystal contacts in both structures are shown in green. N and C indicate the position of the C- and N-termini of one monomer; primed letters denote the termini for the second monomer.
protein atoms in both monomers from symmetry related protein molecules. Interestingly, methylated lysine 202 from monomer A contacts the same residue from monomer B in a symmetry related molecule. In Figure 7, the location of the Ca atoms from residues taking part in crystal contacts for both the native and methylated structure is shown as colored spheres. Different colors were used for various categories of contacts. Yellow and red spheres are for contacts observed only in crystals of the methylated protein, while blue spheres are found only in the crystals of the native protein. Contacts found in both crystal forms are shown as green spheres. Figure 7 illustrates that crystals of the methylated Alr contain more residues taking part in crystal contacts, and as noted above, modified lysine residues, shown as red spheres, make many of these crystal contacts. In this case of Alr, reductive methylation does change the protein surface in a way to promote the formation of a more extensive and apparently more ordered crystalline lattice than that found for the native crystals.

The surfaces of the modified and native Alr crystals are also different in terms of metal and halide content. Four magnesium and three chloride ions were found on the surface of modified Alr and take part in crystal contacts. For the native Alr structure we did not identify any metal or halide ions at equivalent positions. Furthermore, the temperature factors for these surface ions are quite low, with four less than 20 Å², and many are involved in extensive electrostatic interactions. Perhaps the presence of additional metal ions observed exclusively for the methylated crystal form of Alr acts to compensate for the loss of positive charges at the protein surface.

Most importantly, reductive methylation did not alter the overall fold of Alr. Structural alignment of methylated and native Alr shows no significant difference in their overall structures. For the individual monomers the rms difference between their Ca atoms is just under 0.4 Å. Alignment of the active site residues from the two Alr structures shows that reductive alkylation of the enzyme did not result in any significant changes in the position and hydrogen bond pattern of active site residues and the PLP co-factor. Moreover, the hinge angle between N- and C-terminal domains is very similar for both modified and unmodified Alr. Thus, the hinge angles observed for Alr are inherent to this particular enzyme and not an artifact of crystallization. As an aside, this observation makes a strong argument that the disparate hinge angles observed for other Alrs are not a consequence of divergent crystal packing.

Reductive methylation also did not significantly alter the dimer interface, which is found to be comparable between methylated and unmethylated structures (3600 Å² vs. 3500 Å², respectively). For the modified structure, two methylated lysines contribute atoms from their methyl groups to the interface; Mly182 and Mly255. The corresponding lysines in the native structure are not considered to be part of the interface; Lys182 displays poor density and did not have its complete side chain modeled in the native structure and no atoms from Lys255 in one monomer are in contact distance to atoms in the other monomer.

Only two lysines escaped methylation in the modified crystal structure, Lys41 and Lys260 [46]. Lys41 is found covalently bound to the PLP co-factor. Thus its NH group is not a primary amine and is not surprising that this residue is unaffected by the reductive methylation protocol. Lys260 is the lysine residue least exposed to the solvent and it makes hydrogen bonds to Gly137 and Arg138 which, in turn, hydrogen bonds to the phenolic oxygen of the PLP cofactor and to the substrate (see above). These two residues are, therefore, involved in either a covalent bond or a strong polar interaction in the present structure and thus predictably escaped reductive methylation.

**Conclusion**

In conclusion, we report the high-resolution crystal structure of alanine racemase from the dal1 gene of B. anthracis and characterize it kinetically and in an E. coli complementation system. This structure contains some unique features in its active site including a structural chloride atom. It shares a similar hinge angle to its close relative from Geobacillus and has an active site and topology much like other members of this family. Based on the results shown here the active site of Alr is as accessible for inhibitor binding as other alanine racemases studied to date. Furthermore, it is very likely that alanine racemase inhibitors like D-cycloserine or alanine phosphonate will be effective as modulators of sporulation. Finally, as treatment of spores will take place in the environment and not internally, the problems associated with non-specific PLP inhibition ascribed to these inhibitors should not detract from their usefulness in bioremediation. We look forward to exploring more structural studies on these inhibitors as they become available.

**Methods**

**Amplification and cloning of the B. anthracis alr genes**

Two putative open reading frames, dal1 and dal2, for alanine racemase from B. anthracis were identified through sequence comparisons using the known alanine racemase sequence from G. stearothermophilus [22] as a probe against the B. anthracis genome deposited in GenBank [36]. Two sets of primers were used in PCR to amplify the two putative alr genes from genomic DNA of B. anthracis (Ames), dal1–5′ (5′-GGG CCC ATG GAA GAA GCA CCA TTT TAT CGT G-3’)/dal1–3′ (5′-CCC CCT CGA...
GTATCGTCAATATATTACTC-3') and dal2-5' (5'-GGG GCA TAT GAG TTT GAA ATA TGG AAG AG-3')/ dal2-3' (5' CCCCTCGAGAATTCCGTAAGTTTAAAGGAC 3'), resulting in ampiclons of 1169 bp and 1175 bp, respectively. The PCR products were sequenced, inserted into a modified pET28 vector (pET28-TEV) containing a C-terminal His-tag and a TEV protease cleavage sequence, LEENLYFQ/SQVEH, and cloned in E. coli MB1547. (f) denotes the location of the cleavage site.

**Complementation analysis**
Characterization of the two cloned genes continued with their transformation into the D-alanine auxotrophic E. coli strain MB2795 [38]. A plasmid encoding the cloned P. aeruginosa DadX alanine racemase, pMB1921 [49], was used as a positive control. Plasmid pET28-TEV without any inserts served as the negative control. Cells were grown on solid LB medium with and without D-alanine supplementation, and scored for colony growth after 16 h at 37°C as described previously [49].

**Dall overexpression and purification**
Cultures of E. coli BL21(DE3), pLysS containing the pET28-TEV-dal expression plasmids were grown at 37°C in LB medium containing 100 μg/ml kanamycin and 30 μg/ml chloramphenicol to an OD<sub>600</sub> of 0.8. Expression of recombinant proteins was induced by addition of 0.5 mM IPTG and carried at 30°C for 19 hours. Cells were harvested by centrifugation and the cell lysate was cleared by centrifugation at 14,000 rpm and filtered using 0.02 μm Whatman Anotop filters prior to recording data. All measurements were made at 298 K using the DynaPro system according to the manufacturer's instructions (Wyatt Technology).

**Dynamic light scattering**
Purified Alr<sub>Bax</sub> was dialyzed against 20 mM Tris pH 8.0. Protein samples (1 mg/ml) were centrifuged (10 min. at 14,000 rpm) and filtered using 0.02 μm Whatman Anotop filters prior to recording data. All measurements were made at 298 K using the DynaPro system according to the manufacturer's instructions (Wyatt Technology).

**Enzyme Kinetics and Crystallization**
The kinetic parameters (K<sub>e</sub> and V<sub>max</sub>) for the racemization reaction (D- to L-alanine) catalyzed by Alr<sub>Bax</sub> were estimated using the spectrophotometric alanine racemase assay as described previously [40]. Alr<sub>Bax</sub> crystallization screening trials were performed using the vapor diffusion method with sitting drops (5 μl of protein at 15 mg/ml and 5 μl of mother liquor) in 24-well plates incubated at 4°C. Initial screens revealed thin needle crystals growing in 20% PEG 8000, 0.2 M sodium acetate, 0.1 M sodium cacodylate, pH 6.5 [50]. Crystals were optimized using streak-seeding with crushed crystals and further optimized using additive screening resulting in rectangular, deep yellow crystals suitable for data collection. The final crystallization condition was 18% PEG 8000, 0.2 M sodium acetate, 0.1 M sodium cacodylate, pH 6.5, 0.01 M GSH (L-glutathione reduced), 0.01 M GSSG (L-glutathione oxidized).

**Data Collection and Processing**
Crystals were passed through cryoprotectant solutions consisting of 20.7% PEG 8000, 0.2 M sodium acetate, 0.1 M sodium cacodylate supplemented with 3, 6, 9, 12, 15 and 18% (v/v) ethylene glycol, mounted into a nylon loop and flash frozen in liquid nitrogen at 110 K. A native data set was collected at 110 K on a Micromax 007 Hf rotating-anode X-ray generator equipped with a copper anode, Hi-res optics, an RAXIS IV++ image-plate detector (Rikagu) using a frame width of 0.5° and an exposure time of 600 s. Images were integrated using MOSFLM [51], processed with SCALA [52] and analyzed using programs from the CCP4 suite [53]. Data collection and processing statistics for the native data set can be found in Table 1. Alr<sub>Bax</sub> crystallized in space group P2<sub>1</sub> with unit cell parameters a = 49.62 Å, b = 141.27 Å, c = 60.12 Å and β = 103.11. There is one Alr<sub>Bax</sub> dimer per asymmetric unit.

**Structure Determination and Refinement**
Molecular replacement was carried out with MolRep [54] using the G. stearothermophilus Alr (PDB entry – 1SFT) atomic coordinates [22]. Molecular replacement was performed assuming two monomers per asymmetric unit as suggested by a Matthew's coefficient of 2.35 [55] and resulted in the proper orientation of the search model in the crystal lattice (R<sub>free</sub> 43.6%; score 0.699). The primary sequence of the search model was changed to that of Alr<sub>Bax</sub> using Coot [56]. All structural refinements (32.79 – 1.95 Å) were carried in Refmac5 [57] using standard restraints and were followed by visual inspection of protein models and density maps in Coot. Ten cycles of positional refinement, performed using NCS restraints, resulted in R and R<sub>free</sub> of 23.9 and 27.2%, respectively. Waters were added using the arp_water function on Refmac5, and when the active site density was clearly interpretable, PLP was added to both active sites. A further 10 cycles of positional and B<sub>iso</sub> refinements brought R and R<sub>free</sub> to 19.6 and 23.7%, respectively. Water molecules with B-factors higher than 55.0 Å<sup>2</sup> and electron density lower than 1.0 σ on a 2F<sub>obs</sub> – F<sub>calc</sub> map were then deleted.

**TLS Refinement**
B. anthracis crystals displayed somewhat anisotropic x-ray diffraction and previous alanine racemase structures have
shown indication of subdomain movement. This encouraged us to try TLS refinement [58]. TLS analyses were carried on with different domains of the protein acting as a rigid body. All models resulted in similar improvements in R and Rfree, and in the end we adopted the most parsimonious one, which treated all protein atoms found in the asymmetric unit as a rigid body. After TLS refinement, the R and Rfree were 16.0 and 20.1% with root-mean-square deviations from ideality for bond lengths of 0.017 Å and angles of 1.46° (Table 1). As noted above, inclusion of the C-terminal His-tag has resulted in eight additional residues in our sequence. In the final map we attempted to build some of these residues into extra density at the C-terminus, but as we did not gain anything in terms of R or Rfree we have elected to leave out the extra residues from this region in the final structure.

Structure factors and final atomic coordinates for Alr
\( \text{Alr}_{\text{Ala}} \) have been deposited in the Protein Data Bank (PDB ID 3ha1).

**Structural comparisons**

The structure of Alr
\( \text{Alr}_{\text{Ala}} \) was compared to other closely related enzymes; their accession numbers are: 1sf – Alr
\( \text{Gal} \) bound with acetate [22]; 1vfh – Alr
\( \text{Gal} \), with no ligand [27]; 2vd8 – methylated Alr
\( \text{Ala} \) [28], 1rcq – DdX
\( \text{Ala} \) [23] and 1xgc – Alr
\( \text{Ala} \) [26]. Structural alignments were performed using SSQM [59]. Interface surface area was calculated using PISA [60]. The number of polar contacts (hydrogen bonds and salt bridges) was determined using WHAT IF [61,62].

**List of Abbreviations**

Alr: alanine racemase; Bac: *Bacillus anthracis*; DCS: D-cycloserine; Gst: *Geobacillus stearothermophilus*; Mtb: *Myco- bacterium tuberculosis*; Pao: *Pseudomonas aeruginosa*; PLP: pyridoxal 5’-phosphate; rms: root mean square; Sla: *Streptomyces lavendulae*.

**Authors’ contributions**

RC performed research, helped draft manuscript, analyzed results, and prepared figures. US performed research, helped draft manuscript, analyzed results. MD performed research, helped draft manuscript, and analyzed results. RH helped analyze structure and helped prepare figures. KK designed research, analyzed results, helped draft manuscript. All authors read and approved the final manuscript.

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