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**Title:** Another Brick in the Wall: the role of the actinobacterial cell wall in antibiotic resistance, phylogeny and development  
**Issue Date:** 2019-03-20
Structural and functional characterization of the alanine racemase from *Streptomyces coelicolor* A3(2)

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This Chapter is published as:
*Biochemical and Biophysical Research Communications* (2017) Vol. 483, Issue 1, 29 January 2017, Pages 122-128

Supplemental material available at:
https://ars-els-cdn-com.ezproxy.leidenuniv.nl:2443/content/image/1-s2.0-S0006291X16322550-mmc1.pdf
ABSTRACT

The conversion of L-alanine (L-Ala) into D-alanine (D-Ala) in bacteria is performed by pyridoxal phosphate-dependent enzymes called alanine racemases. D-Ala is an essential component of the bacterial peptidoglycan and hence required for survival. The Gram-positive bacterium *Streptomyces coelicolor* has at least one alanine racemase encoded by the putative gene *alr*. Here, we describe an *alr* deletion mutant of *S. coelicolor* which is completely dependent on D-Ala for growth and shows an increased sensitivity to the antibiotic D-cycloserine. The crystal structure of the alanine racemase (Alr) was solved with and without the inhibitors D-cycloserine and propionate. The crystal structures revealed that Alr is a homodimer with residues from both monomers contributing to the active site. The dimeric state of the enzyme in solution was confirmed by gel filtration chromatography, with and without L-Ala or D-cycloserine. Specificity of the enzyme was 66±3 U mg\(^{-1}\) for the racemization of L- to D-Ala, and 104±7 U mg\(^{-1}\) for the opposite direction. Comparison of Alr from *S. coelicolor* with orthologous enzymes from other bacteria, including the closely related D-cycloserine-resistant Alr from *S. lavendulae*, strongly suggests that structural features such as the hinge angle or the surface area between the monomers do not contribute to D-cycloserine resistance, and the molecular basis for resistance therefore remains elusive.
INTRODUCTION

All canonical, proteinogenic amino acids, with the exception of glycine, have a stereocenter at the Cα and can exist either as the L- or D-enantiomer. While in the past it was generally accepted that only L-amino acids had a role in living organisms, studies revealed a variety of roles for free D-amino acids, for example in the regulation of bacterial spore germination and peptidoglycan structure (Cava et al., 2011). Peptidoglycan is essential for cell shape and osmotic regulation and its biosynthesis is dependent on the availability of the less naturally abundant D-alanine (Lam et al., 2009, Steen et al., 2005, Vollmer et al., 2008a). D-alanine (D-Ala) is generated through racemization of the abundant L-enantiomer (L-Ala) by the enzyme alanine racemase (Walsh, 1989).

Alanine racemases (Alr; E.C. 5.1.1.1) are conserved bacterial enzymes that belong to the Fold Type III of pyridoxal phosphate (PLP)-dependent enzymes. Crystallographic studies of Alr’s from different bacterial species revealed a shared, conserved fold consisting of an eight-stranded α/β barrel at the N-terminal domain and a second, C-terminal domain mainly consisting of β-sheets. The PLP cofactor is bound to a very conserved lysine residue at the N-terminal side of the last helix of the barrel. In all studies, Alr’s crystals consist of homo-dimers, with residues from both monomers participating in the formation of the active site. Nevertheless, in-solution studies indicated an equilibrium between monomeric and homo-dimeric states in solution, with the homo-dimer being the catalytically active form (Ju et al., 2011, Strych et al., 2007).

Streptomyces coelicolor is the most widely studied member of the Streptomyces, which are Gram-positive bacteria with a multicellular mycelial life style that reproduce via sporulation (Claessen et al., 2014). They are of great importance for medicine and biotechnology, accounting for over half of all antibiotics, as well as for many anticancer agents and immunosuppressants available on the market (Hopwood, 2007, Barka et al., 2016). Here we describe the heterologous expression, purification and crystal structure of S. coelicolor Alr, encoded by alr (SCO4745). An alr null mutant of S. coelicolor depends on exogenous D-Ala for growth. The purified enzyme catalyzed the racemization of L-Ala to D-Ala in vitro and was shown to be a dimer from gel filtration, both in the absence and presence of L-Ala or the inhibitor D-cycloserine (DCS). Furthermore, the crystal structure of the enzyme has been solved both in the absence and in the presence of the inhibitors DCS and propionate. The comparison of the Alr enzymes from S. coelicolor and its close relative the DCS-resistant Streptomyces lavendulae (Noda et al., 2004b) questions the structural role of Alr in DCS resistance.
MATERIALS AND METHODS

**Streptomyces coelicolor** knock-out mutant and genetic complementation-

*Streptomyces coelicolor* (A3(2) M145 was obtained from the John Innes Centre strain collection and cultured as described (Kieser et al., 2000). *S. coelicolor* alr deletion mutants were constructed according to a previously described method (Swiatek et al., 2012). The -1238/-3 and +1167/+2378 regions relative to the start of *alr* were amplified by PCR using primer alr_LF and alr_LR, and alr_RF and alr_RR (Table S2) as described (Colson et al., 2007). The left and right flanks were cloned into the unstable, multi-copy vector pWHM3 (Table S1) (Vara et al., 1989), to allow for efficient gene disruption (van Wezel et al., 2005). The apramycin resistance cassette *aac(3)IV* flanked by *loxP* sites was cloned into the engineered XbaI site to create knock-out construct pGWS1151. Media were supplemented with 1 mM D-Ala to allow growth of *alr* mutants. The correct recombination event in the mutant was confirmed by PCR. For genetic complementation, the -575/+1197 region (numbering relative to the *alr* translational start codon) encompassing the promoter and coding region of SCO4745 was amplified from the *S. coelicolor* M145 chromosome using primer 4745FW-575 and 4745RV+1197 (Table S2) and cloned into pHJL401 (van Wezel et al., 2000a). pHJL401 is a low-copy number shuttle vector that is very well suited for genetic complementation experiments (van Wezel et al., 2000b).

**Cloning, protein expression and purification**

The *alr* gene (SCO4745) was PCR-amplified from genomic DNA of *Streptomyces coelicolor* using primers alr-FW and alr-RV (Table S2), and cloned into pET-15b with a N-terminal His$_6$ tag. The construct was transformed into *E. coli* BL21 Star (DE3)pLysS electrocompetent cells (Novagen). *E. coli* cultures were incubated in LB medium at 37°C until an OD$_{600}$ of 0.6 and gene expression induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by overnight incubation at 16°C. His$_6$-tagged Alr was purified using a pre-packed HisTrap FF column (GE Healthcare) as described (Mahr et al., 2000). Briefly, after binding of the protein, the column was washed with ten column volumes of 20 mM sodium phosphate buffer, pH 7.0, 500 mM NaCl and 50 mM imidazole and N-terminally His$_6$-tagged Alr eluted with the same buffer but containing 250 mM imidazole. Fractions containing the Alr were desalted using a PD-10 column (GE Healthcare) to remove the imidazole and Alr purified further by gel-filtration using a Superose-12 column (GE Healthcare). The collected fractions were analyzed by SDS-PAGE and those containing pure Alr were pooled, concentrated using a Centriprep centrifugal filter unit (10 kDa cut-off, Millipore), and flash-frozen in liquid nitrogen.

**Crystallization conditions and data collection**

Purified Alr was concentrated to 20 mg mL$^{-1}$ and crystallization conditions were screened by sitting-drop vapor-diffusion using the JCSG+ and PACT premier (Molecular Dimensions) screens at 20°C with 500-nL drops. The reservoir (75 μL) was pipetted by a Genesis RS200 robot (Tecan). Drops were made by an Oryx6 robot (Douglas Instruments). After 1 day, crystals grew in condition number 2-38 of PACT premier, which consisted of 0.1 M BIS-TRIS propane, pH 8.5, 0.2 M NaBr, 20% (w/v) PEG3350. Bigger crystals were grown in 1-μL drops. Prior to flash-cooling in liquid nitrogen, crystals were soaked in a cryosolution consisting of mother liquor, 10% glycerol and 10, 5, or 1 mM inhibitor to obtain the DCS- and propionate-bound
structures.

X-ray data collection was performed at the ESRF (Grenoble, France) on beamline ID23-1 (Kabsch, 2010) using a PIXEL, Pilatus_6M_F X-ray detector. A total of 1127 frames were collected for the native Alr, with an oscillation of 0.15°, an exposure time of 0.378 s, total 426.006 s. The data set was processed by XDS (Kabsch, 2010) and scaled by AIMLESS (Evans & Murshudov, 2013) to a resolution of 2.8 Å. For the DCS-bound Alr 960 frames were collected, with an oscillation range of 0.15°, an exposure time of 0.071 s per image and a total time of 68.16 s. For the propionate-bound structure, 1240 images were collected, with an oscillation degree of 0.1°, an exposure time per image of 0.037 s, and a total time of 45.88 s. Both inhibitor-bound data sets were auto-processed by the EDNA Autoprocessing package in the mxCuBE (Gabadinho et al., 2010) to a resolution of 1.64 Å and 1.51 Å for the DCS-bound and propionate-bound Alr, respectively. The structures were solved by molecular replacement with MOLREP (Vagin & Teplyakov, 1997) using 1VFH as a search model from the CCP4 suite (Winn et al., 2011) and iteratively refined with REFMAC (Murshudov et al., 2011). Manual model building was done using Coot (Emsley & Cowtan, 2004).

**Enzyme kinetics**

The racemization activity of Alr was determined by quantifying the derivatization product of L- and D-Ala by HPLC, as previously described (Hashimoto et al., 1992, Ju et al., 2005). The derivatization reagent consisted of a methanol solution of 10 mg mL⁻¹ o-phthalaldehyde (OPA, Sigma-Aldrich) and 10 mg mL⁻¹ Boc-L-Cys (Sigma-Aldrich). A 0.4 M boric acid solution was adjusted to pH 9.0 with sodium hydroxide. The reaction mixture consisted of 20 µL of 20 mM sodium phosphate buffer, pH 7.0 containing varying amounts of L- or D-Ala from 281 µM to 20 mM. The reaction was started by adding 100 nM of enzyme and stopped after 10 min at 25°C by adding 20 µL of 2 N HCl. The reaction mixture was neutralized by NaOH. After adding 350 µL of boric acid buffer and 100 µL of derivatization reagent, derivatization was conducted for 5 min at 25°C. The fluorescent products were separated on a Kinetex EVO C18 column (Phenomenex) by a gradient from 24% to 46.75% of acetonitrile in water in 30 min and detected by a Shimadzu RF-10AXL fluorescence detector.
RESULTS AND DISCUSSION

Creation of an \textit{alr} null mutant of \textit{S. coelicolor}

To study the role of \textit{alr} in amino acid metabolism and in morphogenesis of \textit{S. coelicolor}, a deletion mutant was created by removal of the entire coding region (see Materials & Methods). In line with its expected role in biosynthesis of the essential D-amino acid D-Ala, \textit{alr} null mutants depended on exogenously added D-Ala (50 µM) for growth (Fig 1a). Re-introducing a copy of the \textit{alr} gene via plasmid pGWS1151 complemented the mutant phenotype, allowing growth in the absence of added D-Ala (Fig 1a).

![Figure 1. SCO4547 encodes alanine racemase Alr. (A) A S. coelicolor alr null mutant fails to grow in the absence of added D-Ala. This phenotype is rescued by the introduction of plasmid pGWS1151 which expresses Alr. Strains were grown for 5 days on SFm agar plates. (B) Rescue of an S. coelicolor alr null mutant with D-Ala produced \textit{in vitro} by Alr. Alr (0.2 nM) was incubated with 10 mM L-Ala for 0, 1, 5, 15, 30 or 60 min, followed by heat inactivation. The reaction mixture was then added to MM agar in 24 well plates and growth of the alr null mutant assessed. As a control, the same experiment was done with heat inactivated Alr (Alr*). Note the restoration of growth to the alr mutant after addition of the reaction mixture with active Alr, but not with heat-shocked Alr. The bottom rows show control experiments with added D-Ala or L-Ala (ranging from 0-1 mM).]
**Alr is the only alanine racemase in *S. coelicolor***

The Alr from *S. coelicolor* (Alr\textsubscript{Sco}) shares 74.9% aa sequence identity with the Alr from the DCS-producing *Streptomyces lavendulae* (Alr\textsubscript{Sla}) (Noda et al., 2004b) and 37.9% with the well-studied Alr from *Geobacillus stearothermophilus* (Alr\textsubscript{Gst}) (Shaw et al., 1997) (Fig. 2). To analyze the activity and kinetics of the enzyme, recombinant His\textsubscript{6}-tagged Alr was expressed in *E. coli* BL21 Star cells and purified to homogeneity (Materials and Methods). Around 45 mg of pure Alr was obtained from 1 L of bacterial culture and Alr was identified using mass spectrometry (Table S4).

To establish if Alr\textsubscript{Sco} converts L-Ala into D-Ala, we designed a combined *in vitro* and *in vivo* assay, whereby 10 mM L-Ala was incubated with 0.2 nM purified Alr-His\textsubscript{6} for 10 sec to 60 min, after which the enzyme was heat-inactivated and the mixture was added to minimal media (MM) agar plates, followed by plating of 10\textsuperscript{6} spores of the *alr* null mutant and incubation of 5 days at 30°C (Fig. 1b). If Alr converts L-Ala into D-Ala, the reaction should generate sufficient D-Ala to allow restoration of growth to *alr* null mutants. Indeed, sufficient D-Ala was produced to allow growth of the *alr* null mutant, whereby biomass accumulation was proportional to the incubation time, while a control experiment with extracts from heat-inactivated Alr did not give any growth (Fig. 1b). Addition of D-Ala also restored growth of *alr* mutants, while no growth was seen for cultures grown in the presence of added L-Ala. Taken together, this shows that no Alr activity is present in *S. coelicolor alr* mutants, and that Alr actively converts L-Ala into D-Ala *in vitro*.

The kinetic parameters of recombinant Alr\textsubscript{Sco} were determined using both L- and D-Ala as substrates. The enzyme shows a K\textsubscript{m} of 6.3 mM and 8.9 mM towards L- and D-Ala, respectively (Table S5). For comparison, kinetic parameters of Alr\textsubscript{Sla} (Noda et al., 2004b) and Alr\textsubscript{Gst} are also shown in Table S5. To analyze possible multimORIZATION of Alr\textsubscript{Sco}, analytical gel filtration was used, which established an apparent molecular weight for the protein of 83 kDa in solution both in the absence and in the presence of L-Ala and DCS (Figure S1); this corresponds roughly to two Alr proteins (43.4 kDa per subunit). These data suggest that Alr\textsubscript{Sco} forms a dimer in solution, both in the presence and in the absence of ligands.
Figure 2. Multiple amino acid sequence alignment of the Alr’s from *S. coelicolor* (Sco), *S. lavendulae* (Sla; PDB 1VFT) and *G. stearothermophilus* (Gst; PDB 1XQK). The sequence alignment showing structural elements of AlrSco was generated with ESPript3.0. α-helices are shown as large coils labeled α, 3₁₀-helices are shown as small coils labeled η, β-strands are shown as arrows labeled β and β-turns are labeled TT. Identical residues are shown on a red background, conserved residues are shown in red and conserved regions are shown in boxes.
Overall structure of Alr from *Streptomyces coelicolor*

The crystal structure of ligand-free Alr<sub>Sco</sub> was determined to a resolution of 2.8 Å (Table S6). The overall structure is similar to that of other prokaryotic Alr proteins (Noda et al., 2004b, Shaw et al., 1997). The asymmetric unit contains four protein molecules (A-D), which interact in a head-to-tail manner to form two dimers (dimer A-B and C-D, Fig. 3a). Each monomer has two structurally distinct domains. The N-terminal domain, comprising residues 1-259, has an eight-stranded α/β-barrel fold, typical of phosphate-binding proteins. The C-terminal domain, residues 260-391, contains mostly β-strands (Fig. 3b). 96% of the amino acids are in the preferred regions of the Ramachandran plot, 4% are in the allowed regions and no residues are in the unfavored areas. After refinement, the r.m.s. deviation between Cα’s of the two interacting molecules A and B is 0.0794 Å, and between C and D is 0.1431 Å.

![Figure 3. Ribbon representation of the Alr from *S. coelicolor*. (a) The asymmetric unit contains two homo-dimers. The two chains within one dimer are colored in gold and dark cyan. (b) The N-terminal domain (1-259) is a α/β-barrel. The C-terminal domain (260-391) mostly comprises β-strands. The catalytic Tyr283 is highlighted in red.](image)

There are two active sites per dimer, which are located at the interface between each α/β-barrel of one subunit and the C-terminal domain of the other. The catalytic core (Fig. 4a) consists of the PLP cofactor, a Lys, and a Tyr, which is contributed by the other subunit. The PLP is bound through an internal aldimine bond to the amino group of Lys46, located at the C-terminal side of the first β-strand of the α/β-barrel. The side chain of the catalytic Lys46 points out of the α/β-barrel, towards the C-terminal domain of the interacting subunit, and in particular, towards Tyr283'. The phosphate group of the PLP is stabilized by hydrogen bonds with the side chains of Tyr50, Ser222 and Tyr374, and with the backbone of Gly239, Ser222 and Ile240. The pyridine ring of the PLP is stabilized by a hydrogen bond between the N-1 of the cofactor and Ne of Arg237. The C2A of the PLP also interacts with oxygen Q1 of the carboxylated Lys141. All residues stabilizing the PLP cofactor (Tyr50, Ser222, Gly239, Ile240, Arg237, Tyr374) are conserved among Alr’s (Shaw et al., 1997). However, the Alr<sub>Sco</sub> lacks one important hydrogen bond between Arg148 and the phenolic oxygen of the PLP molecule, as already observed for the Alr<sub>Sla</sub> (Noda et
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\textit{al.}, 2004a) (Noda \textit{et al.}, 2004b). Among the residues involved in PLP-stabilization, Tyr374, which corresponds to Tyr354 in the well-studied Alr\textsubscript{Gst}, is particularly inter
esting. In fact, while always regarded as a PLP-stabilizer, Tyr354 was shown to be actually anchored by the hydrogen bond with the PLP so to ensure substrate specific
ity of the Alr (Patrick \textit{et al.}, 2002).

\textbf{Figure 4.} Active site of the Alr without ligands (a) and in complex with propionate (b) and D-cycloserine (c). The electron density 2F\textsubscript{o}-F\textsubscript{c} map is shown for the PLP and the two inhibitors. The residues in the active site which are contributed from the monomer not bound to the PLP are indicated as primed numbers. Dotted lines indicate interatomic distances shorter than 3 Å.

\textbf{Alr crystal structures bound to propionate and DCS}

The structure of Alr\textsubscript{Sco} was solved in the presence of the two inhibitors propionate (Morollo \textit{et al.}, 1999) and DCS (Noda \textit{et al.}, 2004b) to a resolution of 1.51 Å and 1.64 Å, respectively. In Alr\textsubscript{Sco}, the carboxylic group of propionate binds in the same orientation in all four active sites present in the crystal unit. One of the carboxylic oxygens of propionate forms hydrogen bonds with the amino group of Met331\textsuperscript{`}, the phenolic oxygen and CE2 of Tyr283\textsuperscript{`} and a water molecule, which bridges the sub
strate to several other water molecules in the active site. The second carboxylic oxygen of propionate interacts with Nz of Lys46, C4A of PLP, the phenolic oxygen of Tyr283\textsuperscript{`} and a water molecule. The electron density around C3 of propionate shows that this atom can have several orientations in the active site. In fact, it can either interact with O4 of the phosphate of PLP, the phenolic oxygen of Tyr283\textsuperscript{`} and a water molecule, or with the phenolic oxygen of Tyr50, the CE of Met331\textsuperscript{`}, Nz of Lys46 and C4A of PLP (Fig. 4b). Both in Alr\textsubscript{Sco} and Alr\textsubscript{Gst} the hydrogen-bonding network stabi
lizing the propionate is very conserved. As argued by (Morollo \textit{et al.}, 1999), the propionate-bound form of the Alr can be considered a mimic of the Michaelis complex formed between the enzyme and its natural substrate L-Ala.

DCS is an antibiotic produced by \textit{S. lavendulae} and \textit{Streptomyces garyphalus}, which covalently inhibits Alr (Noda \textit{et al.}, 2004b, Fenn \textit{et al.}, 2003, Asojo \textit{et al.}, 2014, Priyadarshi \textit{et al.}, 2009, Wu \textit{et al.}, 2008, Lambert & Neuhaus, 1972). The structure of the Alr\textsubscript{Sco} in complex with DCS shows that the amino group of the inhibitor replaces the Lys46 in forming a covalent bond with the PLP C4 (Fig. 4c). The nitrogen and oxygen atoms in the isoxazole ring form hydrogen bonds with Tyr302\textsuperscript{`}, Met331\textsuperscript{`} and a water molecule. The hydroxyl group of the DCS ring also interacts with Met331\textsuperscript{`} and Arg148 (Fig. 4c). The catalytic Tyr283\textsuperscript{`} is at hydrogen-bond distance from the amino group of DCS. DCS molecules superpose well in three of the active sites of the crys-
tal (A, B and C). However, the DCS-PMP adduct in the fourth site showed a shift of
the DCS compared to the other sites, while PLP still superposed well. Comparison
of the hydrogen bonds in the proximity of the active site in the free and in the DCS-
bound form of Alr, revealed a role for Arg148 in substrate stabilization. In fact, while
the side chain of Arg148 is hydrogen-bonded to Gln333 in free Alr, it shifted towards
the hydroxyl moiety of the DCS upon binding of the inhibitor. Gln333 was stabilized
by interaction with carboxylated Lys141. The rearrangement of the hydrogen bonds
involving Arg148, Gln333 and Lys141 does not occur in the propionate-bound struc-
ture, suggesting that Arg148 is involved in stabilization of the carboxylic group of the
substrate.

Alr and DCS resistance

Alr<sub>Sco</sub> shows high sequence and structural similarity to Alr<sub>Sla</sub>. Based on crystallo-
graphic studies, Alr<sub>Sla</sub> was proposed as one determinant of DCS resistance in the
producer strain (Noda <em>et al.</em>, 2004b). However, detailed structural comparison of
different Alr proteins based on interatomic distances between active site residues,
dimerization areas and hinge angles (SI), failed to establish a clear correlation be-
tween structural features and DCS resistance. It therefore remains unclear what the
role of Alr is in DCS resistance in the producer strain.

In conclusion, we showed that SCO4745 encodes the only Alr in <em>S. coelicolor</em>
A3(2) and that it is essential for growth. Our structural studies on Alr<sub>Sco</sub> and compari-
son to AlrSla suggest that Alr is not the only factor that contributes to DCS resistance,
and further investigation is required, in line with recent studies on DCS-producing
Streptomycetes (Matsuo <em>et al.</em>, 2003) and DCS-resistant strains of <em>M. tuberculosis</em>