Binding of non-canonical peptidoglycan controls Vibrio cholerae broad spectrum racemase activity

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Abstract

Broad-spectrum amino acid racemases (Bsrs) enable bacteria to generate non-canonical D-amino acids (NCDAAs), whose roles and impact on microbial physiology, including modulation of cell wall structure and dissolution of biofilms, are just beginning to be appreciated. Here we used a diverse array of structural, biochemical and molecular simulation studies to define and characterize how BsrV is post-translationally regulated. We discovered that contrary to Vibrio cholerae alanine racemase AlrV highly compacted active site, BsrV’s is broader and can be occupied by cell wall stem peptides. We found that peptidoglycan peptides modified with NCDAAs are better stabilized by BsrV’s catalytic cavity and show better inhibitory capacity than canonical muropeptides. Notably, BsrV binding and inhibition can be recapitulated by undigestable peptidoglycan sacculi as it exists in the cell. Docking simulations of BsrV binding the peptidoglycan polymer generate a model where the peptide stems are perfectly accommodated and stabilized within each of the dimer’s active sites. Taking these biochemical and structural data together, we propose that inhibition of BsrV by peptidoglycan peptides underlies a negative regulatory mechanism to avoid excessive NCDAAs production. Our results collectively open the door to use “à la carte” synthetic peptides as a tool to modulate DAAs production of Bsr enzymes.

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1. Introduction

Amino acids exist as both L and D enantiomers, being the L-form the most predominant [1]. Whilst L-amino acids (LAA) are the building blocks of proteins in all kingdoms of life [2], the presence of D-amino acids (DAA) is usually linked to the existence of dedicated amino acid racemases, which are able to interconvert L to D-amino acids and vice versa [3]. The most commonly studied DAAs are the Ala-racemase (Ala-R) and the Glu-racemase (Glu-R) implicated in the synthesis of D-Ala and D-Glu, main components of the bacterial peptidoglycan, also called murein sacculus [4,5]. The bacterial peptidoglycan is an indispensable net-like subcellular structure formed by groups of sugars (N-acetyl-glucosamine-N-acetyl-muramic acid) cross-linked by short peptides chains that include both LAA and DAAs [6]. The archetypical peptide stem structure is L-alanine, D-glutamic acid, a dibasic amino acid (typically meso-diaminopimelic acid or L-lysine), D-alanine, and D-alanine. Therefore, both cytoplasmic racemases Ala-R and the Glu-R, as well as their reaction products (DAA), are fundamental to maintain the murein sacculus structure and, thus, bacterial fitness [6].

Bacteria can have several copies of both these racemases [7]. For example, Vibrio cholerae presents two non-functional redundant...
Ala-R, one of which is primarily related to peptidoglycan biosynthesis. Interestingly, V. cholerae encodes an additional multispecific amino acid racemase, named BsrV for broad-spectrum racemase Vibrio, which produces non-canonical DAAs (NCDAAAs), i.e., DAAs that are different from those usually present in the cell wall [8,9]. BsrV is an Ala-R homolog. As Ala-R, BsrV uses pyridoxal 5-phosphate (PLP) as a cofactor and can efficiently racemize Ala. However, BsrV produces nine additional DAAs, including the non-β-branched aliphatic amino acids (Leu, Met, Ser, Cys, Gln and Asn) and the positively charged amino acids (His, Lys and Arg) [9].

In V. cholerae, expression of BsrV is regulated by the stress sigma factor RpoS in response to high population density and nutrient exhaustion (i.e., stationary growth phase) [8]. In this context, BsrV is expressed, and its activity drives the production and release of millimolar concentrations of NCDAAAs to the extracellular media. NCDAAAs can then be used as substrates by certain cell wall synthetic enzymes to induce chemical changes in the peptidoglycan composition [8,10,11]. It has been demonstrated that such cell wall chemical editing by NCDAA down-regulates peptidoglycan synthesis to enable cell wall adaptation to stationary phase conditions [8,10,11].

In addition to being regulators of peptidoglycan synthesis and integrity [8,10,11], NCDAA has also been reported to be involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16]. As their L-enantiomeric counterparts, the physiologically relevant D-amino acids are involved in diverse cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell activation [16].
were separated with a linear gradient of triethylamine phosphate/acetonitrile in HPLC with an Aeris peptide column (250 × 4.6 mm; 3.6 μm particle size) (Phenomenex, USA) and detected at Abs.340 nm. To determine the inhibition effect of the sacculi in BsrV's activity, 35 μg of sacculi were incubated for 5 min at 37 °C with BsrV and 4 mM of L-Ala in Tris-HCl 50 mM pH 8. The product was revealed with DAAO [21]. DAAO reaction was determined by coupling 10 μL of the extract into 150 μL of a reaction containing: sodium phosphate buffer 100 mM pH 7, Trigonopsis variabilis D-amino acid oxidase (DAAO) (Komarova et al., 2012) 3.6 U/ml, horseradish peroxidase 1 U/mL, o-phenylenediamine (OPD) 2 mg/mL and FAD 3 mg/ml. This two-step assay permits the quantification of H₂O₂ (DAAO is able to produce α-ketoacid, NH₃ and H₂O₂ from DAAO). Peroxidase reduces H₂O₂ releasing free O₂ that reacts with OPD, leading to the production of 2,3–25 diaminophenazine. The reaction was incubated for 2 h at 37 °C and inactivated with HCl 3 M, giving a colorimetric product that can be measured at 492 nm. To determine the inhibition effect of muropeptides in BsrV's activity, 0.1 mM of M4 (GlcNAc-MurNAc-Ala-Glu-DAP (Diaminopimelate)-Ala), M3M (GlcNAc-MurNAc-Ala-Glu-DAP-Met), M3R (GlcNAc-MurNAc-Ala-Glu-DAP-Arg) and D-cycloserine were incubated with BsrV and 4 mM of L-Ala for 5 min at 37 °C (1/40 relation) in Bicarbonate buffer 50 mM pH 9. In the case of tripeptide and D-Ala-D-Ala, equal concentration (amino acid, tripeptide/dipeptide) was utilized. The product was revealed with Marley's reagent as described above.

2.6. Structural determination

Crystallization of BsrV tagless was performed as previously described for the His-tagged proteins [9]. Briefly, a high-throughput NanoDrop ExtY robot (Innovayne Technologies Inc.), the commercial Qiagen screens The JCGS + Suite and The PACT Suite and the Hampton Research screens Index, Crystal Screen and Crystal Screen 2 were used to get crystals by the sitting-drop vapor-diffusion method. Best crystals were obtained with 0.1 M Bis-Tris propane pH 7.5, 0.2 M Sodium Iodide, and 24% (p/v) of PEG 3350. X-ray data collection was performed on the X06SA beamline at the SLS synchrotron-radiation facility in Villigen, Switzerland. Data sets were collected using a PILATUS 6 M detector, and were processed using XDS [27] and scaled using SCALA [28] from the CCP4 suite [29]. The structure was solved by the molecular replacement method with MOLREP [30] from the CCP4 suite using the His-tagged version of BsrV (PDB code 4BEU) as initial model. Refinement was performed with PHENIX [31] and modeling with Coot [32]. The stereochemistry of the models was verified using MolProbity [33]. A summary of the data collection and refinement statistics is given in Table 1.

| Table 1 | Data collection and refinement statistics. |
|---------|-------------------------------------------|
| Wavelength (Å) | 1.0 |
| Resolution range (Å) | 51.2–1.52 (1.57–1.52) |
| Space group | P 2₁ 2₁ 2₁ |
| Unit cell | a, b, c (Å) 54.03, 82.09, 160.21 |
| | z, β, γ (%) 90, 90, 90 |
| Total reflections | 17,752,204 |
| Unique reflections | 111,214 |
| Multiplicity | 7.2 (8.2) |
| Completeness (%) | 99.9 (99.9) |
| Mean I/σ(I) | 14 (4.2) |
| R-merge | 0.092 (0.562) |
| R-pim | 0.036 (0.205) |
| CC1/2 | 0.981 (0.819) |
| Reflections used in refinement | 111,036 (10943) |
| R-work/ R-free | 0.1799/0.1984 |
| Number of non-hydrogen atoms | 6732 |
| macromolecules | 5965 |
| ligands | 44 |
| solvent | 723 |
| Protein residues | 776 |
| RMS bonds (Å) | 0.018 |
| RMS angles (°) | 1.63 |
| Ramachandran favored (%) | 97.40 |
| Ramachandran allowed (%) | 2.60 |
| Ramachandran outliers (%) | 0.00 |
| Average B-factor (Å²) | 20.68 |
| macromolecules | 19.12 |
| ligands | 26.61 |
| solvent | 33.21 |
| PDB code | 7AGZ |

Statistics for the highest-resolution shell are shown in parentheses.

were considered statistically significant, with the following ranking: p < 0.05(*); p < 0.001(**).

2.9. Data availability

The atomic coordinates and structure factors for BsrV His-Tagged less (PDB 7AGZ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, New Jersey, USA (http://www.rcsb.org/). The rest of the data are contained within this manuscript.

3. Results

3.1. BsrV C-terminal His-tag interacts with the enzyme catalytic channel

The crystal structures of His-tagged constructs of BsrV and the broad-spectrum racemase (Bsr) from Aeromonas hydrophila (Bsr_Hy) have been reported earlier [9]. Both enzymes showed a remarkable facility to crystallize [9]. After exhaustive structural analysis of BsrV and Bsr_Hy crystals, we realized that the C-terminal His-tag added to the racemases for purification purposes was tethering the dimers (Fig. S1). This effect was caused by the interaction of the C-terminal His-tags belonging to one dimer with the active site of adjacent dimers and thus increasing crystal contacts. Strikingly, multiple interprotein interactions were observed between Bsr's His-tags and several residues of its catalytic channel (Fig. 1). The interactions were particularly numerous for Bsr_Hy, whose His-tag extended through the entry site almost reaching the PLP in the catalytic site (Fig. 1, Fig. S1). In order to validate this, a His-tag less BsrV protein was crystallized and its structure was solved at atomic resolution (1.52 Å, Table 1). As expected, superimposition of the His-Tag and His-tag less BsrV revealed their strong similarity.
(RMSD: 0.350 Å), which is in concordance with their mirroring biochemical performance (Fig. S2) [8]. Besides minor structural changes (N207, Y208, Y246 and D300) resulting in a small decrease of around 1 Å in the entry site’s aperture, no significant differences were observed in the conformation of the catalytic site (Fig. S2). The fact that Bsr display an unusually large active site [9] capable of binding oligopeptides (His-tag) together with their periplasmic localization made us hypothesize that the stem peptides of the peptidoglycan (muropeptides) might be a more physiological interacting partner of BsrV.

3.2. BsrV binds to cell wall muropeptides

To test this hypothesis, we compared the capacity of BsrV and AlrV (the Ala-R from V. cholerae) to bind muropeptides. We isolated muropeptides from V. cholerae ΔbsrV strain given that digestion of the peptidoglycan of this mutant renders a homogenous pool of canonical muropeptides, does not present any NCDAA-modified muropeptides (Fig. S3). BsrV retained 50–60% of V. cholerae isolated canonical muropeptides compared to AlrV, which retained ~25%. This binding appeared to be nonspecific since similar retention was observed using a control protein (AmpC-His) that does not bind peptidoglycan (Fig. 2A). Remarkably BsrV muropeptide binding increased to a 60–75% when challenged with D-Arg/D-Met muropeptides (Fig. 2A, Fig. S3), suggesting a certain specificity of Bsr for NCDAA-edited peptidoglycan.

To assess the potential fitting of muropeptides bound to BsrV’s active site, we generated docking models for several muropeptides using as template the conformation exhibited by the His-tag that is stabilized within BsrV’s active site in our crystal structure. We then performed molecular dynamic (MD) simulations of BsrV and Alr catalytic pockets occupied by canonical (disaccharide-tetrapeptide, GlcNAc-MurNAc-Ala-Glu-DAP-Ala; M4) and non-canonical (disaccharide-tetrapeptide with terminal D-Met or D-Arg instead of D-Ala; M3M or M3R, respectively) muropeptides (Fig. S3 and movies S1, S2 and S3). These analyses suggested that muropeptides can interact with BsrV’s catalytic channel in a manner analogous to that observed in crystal structures of the His-tagged BsrV and BsrA. These docked complexes show many putative strong polar and hydrophobic stacking interactions between residues from the BsrV active site and all the peptide stem residues. It is noteworthy that the sugar rings (NAG, NAM) of the muropeptides can also establish polar interactions with the loops of BsrV that shape the entry of its active site cavity (Fig. 2BC). In contrast, docking/MD analyses suggested that muropeptides would encounter numerous steric clashes along the Alr active cavity, including the entry site, rendering this interaction very unlikely (Fig. S3). MD simulations also suggest that non-canonical muropeptides (M3M and M3R) bind to the active site of BsrV more strongly than canonical muropeptides establishing numerous hydrogen bonds and strong salt bridges.

Fig. 1. Muropeptide recognition by BsrV. Crystal structure of (A) BsrV and (B) BsrA active site entry. Left, the molecular surface of one monomer is colored in brown (the surface for the partner is omitted for clarity). The C-terminal His-tag (purple sticks) of a crystal partner enters into the BsrV (A) and BsrA (B) active sites. Right, stereoview showing the polar interactions (dotted lines) between the His-tag and the Bsr active site. Catalytic PLP is represented in sticks and labeled. Cl− ion represented as a green sphere. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.3. BsrV is inhibited by NCDAA-modified muropeptides

Since amino acids in muropeptides do not exhibit free amino groups linked to chiral carbons, bound peptides seemed likely to function as non-racemizable competitive inhibitors. To test this possibility, we performed in vitro assays of BsrV capability to racemize d-Ala in the presence or absence of different muropeptides (Fig. 3A). All monomer muropeptides assayed caused a reduction (from ~20% to 65%) in d-Ala production compared to control reactions without muropeptide. In addition, non-canonical muropeptides show a higher degree of inhibition (~65% reduction in d-Ala production) than the canonical M4 (~20% reduction). m-D-cycloserine and short peptides (dipeptide and tripeptide), in general, inhibit the least (Fig. 3A). This result is likely due to the reduced number of potential interactions the small peptides can form compared to longer peptides, differentially affecting their stabilization within the active site cavity (Fig. 2B). Also, cross-linked monomers (D44) did not cause any detectable inhibition suggesting that linear peptides are needed to compete for the active site entry. Collectively, these analyses suggest that the production of NCDAA modified muropeptides as result of BsrVs racemization of LAA might also modulate its activity in vivo. To further explore this possibility, we assessed whether undigested V. cholerae sacculi also exhibits inhibitory properties (Fig. 3B). When BsrV is incubated with canonical and NCDAA modified sacculi, we observed a significant reduction in BsrV dependent d-Ala production in the...
The presence of NCDAAs-free sacculi (AbsrV peptidoglycan), which further decreased when sacculi containing NCDAAs (9% of total muropeptides) were used instead (Fig. 5A) [10]. In contrast, AlrV’s activity was not reduced by the presence of any type of sacculi, consistent with results from the docking modeling and affinity assays (Fig. 3B).

3.4. BsrV binds polymeric peptidoglycan

Molecular models based on NMR studies suggest that peptidoglycan forms a right-handed helical structure with the peptide stems projecting out at 120° intervals [19]. To better understand the interaction between peptidoglycan and BsrV, we ran docking simulations of peptidoglycan fragments with the BsrV molecule (Fig. 4). Notably, the distance between the active sites of a BsrV dimer and their relative rotation fit well with the peptidoglycan fragment structure reported by Meroueh et al (Fig. 4) [19]. In fact, in this model, the peptide stems from the peptidoglycan fragment are perfectly accommodated and stabilized within each of the active sites of the dimer. This precise molecular fit between the BsrV structure and the two stem peptides radiating from the same active sites of the dimer can be simultaneously occupied with the peptide moieties of a single peptidoglycan -strand separated by one turn. Analyses of enzyme activity, coupled with our modeling assays, suggest that muropeptides can occupy BsrV’s (but not AlrV’s) catalytic site and thereby serve as competitive inhibitors. Thus, our findings raise the possibility that the production of NCDAAs by BsrV and related periplasmic broad-spectrum racemases is down-regulated when peptidoglycan contains sufficient levels of NCDAAs. Such downregulation might reflect global levels of peptidoglycan modification in the cell. Alternatively, it might also function to fine-tune the spatial allocation of NCDAAs by promoting their equal distribution throughout peptidoglycan.

Negative feedback control of BsrV activity by non-canonically modified-peptidoglycan seems reasonable given that NCDAAs reduce peptidoglycan synthesis [8,10,11] and that excessive concentration of NCDAAs can be lethal [18]. According to our model, the synthesis of BsrV will be produced on early stationary phase conditions in a RpoS dependent manner [8]. Following the enzyme’s translocation to the periplasm, production of NCDAAs from the corresponding l-forms ensues (Fig. 4). Since NCDAAs peptidoglycan incorporation appears to be constrained to active murein biosynthetic sites [20], local concentrations of NCDAAs modified muropeptides are likely to become very high, promoting their binding to and inhibition of BsrV. Inactivation of BsrV by NCDAAs-modified muropeptides reduces local production of NCDAAs, establishing a negative feedback loop in which the products of BsrV, preventing over-production of NCDAAs that might ultimately be deleterious [18]. Moreover, in addition to the effect on V. cholerae, fine-tuning BsrV’s activity may also have implications on the physiology of nearby organisms as NCDAAs are known to impact a number of distinct cellular processes such as catabolism [12], biofilm formation [13], bacteria-bacteria interactions [14], microbiome biodiversity [15], modulation of host immune cells, and immune cell response [16].

The ability of BsrV to interact with peptides introduces a number of intriguing additional possibilities for the regulation of broad-spectrum racemases. For example, short linear non-ribosomal peptides (NRP), such as some secreted peptides involved in bacterial communication [21], might also interact with BsrV, either as regulators or as substrates. Given the impact of NCDAAs on a variety of cellular processes [14,15,22], bacteria may have evolved diverse ways to control their production and to regulate its spatiotemporal allocation.
Fig. 4. Proposed model of BsrV regulation by NCDAA in peptidoglycan. (A) In exponential growth phase, *V. cholerae* does not express BsrV and consequently, its peptidoglycan is composed only of canonical muropeptides. In the transition to stationary phase, *V. cholerae* expresses BsrV, an RpoS-dependent, periplasmic, multispecific amino acid racemase [8]. BsrV produces high (millimolar) concentrations of NCDAA that accumulate in the periplasmic space and also pass to the extracellular media [10]. (B) NCDAA are incorporated into peptidoglycan in stationary phase cells. Peptidoglycan containing such modifications is a more potent inhibitor of BsrV than is unmodified peptidoglycan; thus, a negative feedback loop is generated to control BsrV’s activity. (C) Ultimately, high levels of peptidoglycan modification may turn off the majority of BsrV, thereby preventing hyper modification and excessive accumulation of free NCDAA. A detail of Fig. 4 is shown. CS1 and CS2: catalytic sites. (D) BsrV expression shuts down when growth resumes, preventing further production and incorporation into peptidoglycan of NCDAA. (E) Molecular docking of the NMR-peptidoglycan structure [19] with BsrV dimer. Peptidoglycan is drawn in sticks with glycan chains colored in orange and peptide stems colored in magenta. BsrV active sites interact with the peptide moieties of the sacculus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Collectively, our results open the door to use “à la carte” synthetic peptides as a tool to modulate DAAs production of Bsr enzymes. So, the effect of the DAAs in bacterial fitness and biotechnology could be modulated by the usage of diverse peptides that, in turn, would control Bsr activity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

AE, CC-L, NGB, AR-A, and JK conducted the study and FC, JAH and AE wrote the paper. CC-L carefully revised the paper. All authors took part in interpreting the results. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.01.031.

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