HHS Public Access

Author manuscript

Chembiochem. Author manuscript; available in PMC 2021 April 17.

Published in final edited form as:

Chembiochem. 2020 April 17; 21(8): 1155–1160. doi:10.1002/cbic.201900565.

The L-alanosine gene cluster encodes a pathway for diazeniumdiolate biosynthesis.

Tai L. Ng[a],†, Monica E. McCallum[a],†, Christine R. Zheng[a], Jennifer X. Wang[b], Kelvin J. Y. Wu[a], Emily P. Balskus*,[a]

[a]Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford St, Cambridge, MA 02138 (USA)

[b]Small Molecule Mass Spectrometry Facility, Faculty of Arts and Sciences, Division of Science Harvard University, 52 Oxford St, Cambridge, MA 02138 (USA)

Abstract

N-nitroso-containing natural products are bioactive metabolites with antibacterial and anticancer properties. In particular, compounds containing the diazeniumdiolate (*N*-nitrosohydroxylamine) group display a wide range of bioactivities ranging from cytotoxicity to metal chelation. Despite the importance of this structural motif, knowledge of its biosynthesis is limited. Here, we describe the discovery of a biosynthetic gene cluster in *Streptomyces alanosinicus* ATCC 15710 responsible for producing the diazeniumdiolate natural product L-alanosine. Gene disruption and stable isotope feeding experiments identified essential biosynthetic genes and revealed the source of the *N*-nitroso group. Additional biochemical characterization of the biosynthetic enzymes revealed that the non-proteinogenic amino acid L-2,3-diaminopropionic acid (L-Dap) is synthesized and loaded onto a free-standing peptidyl carrier protein (PCP) domain in L-alanosine biosynthesis, which we propose may be a mechanism of handling unstable intermediates generated en route to the diazeniumdiolate. These discoveries will facilitate efforts to determine the biochemistry of diazeniumdiolate formation.

Graphical Abstract

Diazeniumdiolate biosynthesis: L-alanosine is an unusual diazeniumdiolate-containing natural product. The gene cluster that encodes L-alanosine production has been identified and the origin of the *N*-nitroso group has been characterized. This discovery advances understanding of the biological origins of this functional group.

*Corresponding Author balskus@chemistry.harvard.edu.

†These authors contributed equally to this work.

Experimental Section

Complete experimental details are provided in the Supporting Information.

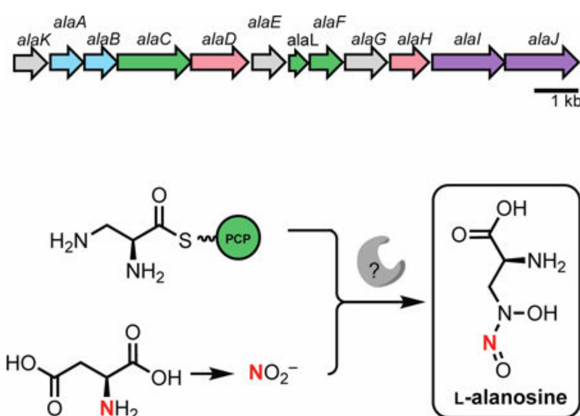
Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript



Keywords

biosynthesis; enzymes; natural products; *N*-nitroso compounds; N-N bond

---

N-nitroso-containing small molecules play prominent roles in human health, disease, and therapeutics development.[1,2] In biological systems, nitric oxide synthases (NOSs) convert L-arginine into L-citrulline and nitric oxide (NO), which can undergo further oxidation to nitrite (NO_2^-) in aqueous systems. Both NO_2^- and NO can react with secondary amines to afford *N*-nitrosamines, which are notorious environmental toxins and carcinogens, yet have been exploited as a bioactive pharmacophore in several *N*-nitrosourea-containing chemotherapeutic drugs.[1,3] While many of these compounds are formed abiotically, the *N*-nitrosourea in streptozotocin (**2**) was recently reported to arise from the action of a metalloenzyme[4] (SznF, Figure 1a), and the diazo functional group in cremeomycin (**4**)[5] is thought to be produced via a transient *N*-nitrosamine intermediate (**3**) generated by an ATP-dependent enzyme (Figure 1b). Recent studies have linked bacterial diazeniumdiolate-containing metabolites to quorum sensing (**6**) and metal-acquisition (**7**), revealing an emerging ecological role for this group of natural products (Figure 1c).[6,7] However, the genetic and biochemical basis for diazeniumdiolate biosynthesis remains poorly understood.

L-alanosine (**5,** Figure 1c) is a naturally occurring diazeniumdiolate-containing amino acid produced by the bacterium *Streptomyces alanosinicus* ATCC 15710 that was discovered in the 1960s from a Brazilian soil sample.[8] **5** has since been studied for its antibiotic, antiviral, and antitumor activities. The unusual diazeniumdiolate group confers metal chelating properties to **5**.[9] This moiety also has been demonstrated to release NO upon metabolism of **5** by L-amino acid oxidases, generating reactive nitrogen species.[10] In the decades following its isolation, **5** has been demonstrated to act as an antimetabolite targeting *de novo* purine biosynthesis and has been investigated in clinical trials to treat various tumors (SDX-102).[11]

Despite numerous studies exploring **5** as a potential therapeutic agent, the biosynthetic gene cluster responsible for its production has not been identified. Recently, the Eberl and Hertweck groups reported the biosynthetic gene clusters for the *N*-nitrosamines fragin (**6**)[6] and gramibactin (**7**),[7] revealing putative enzymes that could install the diazeniumdiolate

Author Manuscript

moiety. HamC, which has been demonstrated to oxidize *p*-aminobenzoic acid to *p*-nitrobenzoic acid *in vitro*, is proposed to mediate N-N bond formation in fragin biosynthesis, but this putative reaction has yet to be verified *in vivo* and *in vitro*. Similarly, the SznF homolog GrbD from the gramibactin pathway is proposed to catalyze N-N bond formation, but its activity has not been demonstrated.[6,12] Elucidating the biosynthesis of **5** would therefore improve our limited insights into enzymatic installation of the diazeniumdiolate, and more broadly the *N*-nitroso group. Here, we report the discovery of the L-alanosine (*ala*) biosynthetic gene cluster (Figure 2a). Using a combination of feeding studies, *in vivo* gene inactivation experiments, and *in vitro* biochemistry, we have revealed a plausible biosynthetic pathway, paving the way for further understanding of *N*-nitroso assembly in living organisms.

We initially hypothesized that **5** could be derived from *N*-hydroxylation and *N*-nitrosation of the putative biosynthetic precursor L-2,3-diaminopropionic acid (L-Dap, Figure 2b), a nonproteinogenic amino acid involved in several siderophore biosynthetic pathways.[13–15] In staphyloferrin B biosynthesis, SbnA uses pyridoxal 5'-phosphate (PLP) as a cofactor to ligate *O*-phospho-L-serine (**8**) and L-glutamate to form a dipeptide *N*-(1-amino-1-carboxyl-2-ethyl)-glutamic acid (**9**) that is cleaved by deaminase SbnB to generate L-Dap (Figure S1).[16] To identify the *ala* gene cluster, we sequenced the genome of *S. alanosinicus* ATCC 15710 and searched for homologs of the L-Dap biosynthetic genes. This strategy revealed a 13.5 kb genomic region that encodes homologs of SbnAB (AlaAB), an amino acid adenylation domain-containing protein (AlaC), a free-standing peptidyl carrier protein (PCP) domain (AlaL), and a thioesterase (TE) enzyme (AlaF) (Figure 2a, Table 1). These biosynthetic enzymes are encoded alongside homologs of the enzymes CreD (AlaJ) and CreE (AlaI), which are known to produce NO_2^- from L-aspartic acid in cremeomycin biosynthesis,[5] a predicted flavin-dependent *N*-hydroxylase (AlaD), and a potential flavin reductase (AlaH) (Figure 2a). The putative *ala* gene cluster also contains a transcriptional regulator (AlaK), a GAF-domain containing protein (AlaE) and a PAS-domain containing protein (AlaG) that likely regulate the transcription of this gene cluster. The *ala* gene cluster boundaries were initially determined using antiSMASH v 5.0.0 with ClusterBlast and the genome neighborhood feature of IMG/JGI (Figure S8). This analysis revealed highly similar gene clusters in a number of additional *Streptomyces* species.

Based on the predicted functions of the enzymes encoded in this cluster, we proposed a biosynthetic hypothesis for the assembly of **5** (Figure 2b). AlaAB would generate L-Dap, which could be loaded onto the phosphopantethienyl (ppant) arm of the free-standing PCP AlaL by A domain AlaC. This biosynthetic logic parallels the proposed pathway for fragin construction in which diazeniumdiolate installation could occur on enzyme-tethered intermediates.[6] The predicted flavin-binding enzyme AlaD and the flavin reductase AlaH could be a two-component flavin *N*-monooxygenase involved in forming *N*-hydroxy-L-Dap (**11**), and an unknown enzyme (potentially one of the remaining proteins encoded by the *ala* gene cluster) could install the *N*-nitroso group using NO_2^- generated by AlaIJ. Notably, the *S. alanosinicus* genome does not encode homologs of any biosynthetic enzymes from the fragin and L-graminine pathways. Furthermore, the genome lacks homologs of SznF and KtzT, recently reported N-N bond-forming enzymes in streptozotocin and piperazate

biosynthesis, respectively.[4,17] Moreover, homologs of other suspected N-N bond-forming enzymes, Spb40/Tri28 from the s56-p1 and triacsin biosynthetic pathways[18,19] and FzmP/KinJ from fosfazinomycin and kinamycin biosynthetic pathways, are also absent.[20] Our bioinformatics analysis therefore suggests that biosynthesis of **5** either employs a novel N-N bond-forming enzyme or generates the diazeniumdiolate functional group non-enzymatically.

To establish the link between the *ala* gene cluster and biosynthesis of **5**, we performed several gene inactivation experiments in *S. alanosinicus*. Production of **5** was abolished when *alaC*, *alaD*, and *alaI* were deleted via the well-established PCR-targeting and λ-red-mediated recombination platform (Figure 3a). This confirms that the activity of the A domain (AlaC), redox chemistry (via AlaD) and NO_2^- generation (via AlaI) are all essential. When the *ΔalaI* mutant was supplemented with NO_2^-, **5** was not detected (Figure 3a, maroon line). This suggests that AlaI may be important for NO_2^- incorporation, or that the deletion of *alaI* has introduced a polar mutation that has impacted a previously undetected regulatory mechanism in the gene cluster. Lastly, the deletion of *alaC* and concomitant loss of **5** imply that the non-ribosomal peptide synthetase (NRPS) machinery is necessary for biosynthesis and may facilitate the generation of an unstable, enzyme-bound intermediate during diazeniumdiolate assembly. Taken together, these gene-inactivation experiments confirmed the role of the *ala* gene cluster in the biosynthesis of **5** and identified several indispensable enzymes for further *in vitro* characterization.

Because the putative *ala* gene cluster encodes homologs of the nitrite-generating enzymes CreD and CreE (AlaJ and AlaI, respectively, Figure 4a), we proposed that the precursor of the distal nitrogen of the *N*-nitroso group is NO_2^-. In addition to its role in non-enzymatic *N*-nitrosation reactions, NO_2^- is used biosynthetically as a source of nitrogen in the formation of other N-N bond linkages, including diazo and hydrazide groups.[20,21] To test this hypothesis, we overexpressed and purified N-His_6-AlaI and N-Strep-AlaJ, and we demonstrated their ability to generate NO_2^- from L-aspartic acid with the necessary cofactors (Figure 4b). We next fed $^{15}NO_2^-$ to cultures of *S. alanosinicus* and observed incorporation of ^{15}N into **5** (~ 80%, Figure 3b). Tandem high resolution-mass spectrometry (HR-LCMS/MS) revealed the distal *N*-nitroso nitrogen is labeled (Figure S2 and S3). Feeding $^{15}NO_3^-$ also resulted in lower enrichment of ^{15}N-labeled **5** (Figure S5); this could potentially arise from conversion of NO_3^- to NO_2^- by nitrate reductases encoded by *S. alanosinicus*. Finally, we also fed ^{15}N-L-aspartic acid, the presumed precursor of $^{15}NO_2^-$ and observed enrichment of ^{15}N-**5** (~ 10%, Figure 3b). Together, these results suggest that the biosynthesis of **5** employs NO_2^- generated from L-aspartic acid, and the distal nitrogen of the diazeniumdiolate is derived from NO_2^-.

Having established the origin of the *N*-nitroso group in **5**, we next sought to confirm the role of L-Dap in this biosynthetic pathway. We first overexpressed and purified *N*-His_6-AlaB for *in vitro* biochemical assays. SbnB, a homolog of AlaB, was previously characterized by performing the L-Dap synthase reaction in reverse to generate **9** (Figure S1).[16] We employed the same strategy to characterize AlaB by incubating the enzyme with L-Dap and NADH. We observed rapid consumption of NADH and production of **9** (Figure 2b, Figure

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

S6 and S7). Therefore, AlaB is a functional homolog of the previously characterized L-Dap synthase component SbnB.[16]

The involvement of a free-standing A domain in the biosynthesis of **5** and its putative loading of L-Dap are unusual, as there are only three examples of biochemically characterized A domains that utilize this amino acid.[22–24] A domain active sites contain 8–10 binding-pocket residues that dictate substrate specificity.[25] Several bioinformatic methods that predict A domain substrate specificity from these amino acid residues have been developed.[26] However, these tools[27] failed to predict L-Dap as the substrate of AlaC. The same is true of other A domains that have been experimentally demonstrated to activate L-Dap (Table S1).

To test if AlaC can recognize and load L-Dap, we overexpressed and purified N-His_6-tagged AlaC and N-His_6-C-His_6-tagged AlaL for *in vitro* biochemical assays. The preferred substrate of AlaC was confirmed to be L-Dap using the ATP-[^{32}P]Pi exchange assay (Figure 5a). The ability of purified apo-AlaL to undergo successful posttranslational modification was determined via incubation with the promiscuous ppant transferase Sfp and the fluorescent coenzyme A (CoA) analog BODIPY-CoA.[28] Finally, we demonstrated that isotopically labeled L-$^{15}N_2$-Dap was activated by AlaC and loaded onto the ppant arm of holo-AlaL using whole-protein mass spectrometry (Figure 5b). Thus, the NRPS machinery encoded in the *ala* gene cluster is capable of activating L-Dap and loading it onto the PCP AlaL. Given that *alaC* was determined to be necessary for the production of **5**, the AlaL-tethered L-Dap aminoacyl thioester **10** (Figure 2b) is likely a biosynthetic intermediate.

In summary, we have identified a set of genes that are required for the biosynthesis of **5** in *S. alanosinicus.* While genetic deletion has confirmed these genes are essential, we cannot currently conclude they are minimally required, as all attempts to heterologous express the *ala* gene cluster in *Streptomyces lividans* TK64 have been unsuccessful. The *ala* gene cluster we have introduced into this heterologous host is flanked by ~9 kb of native genomic DNA. This negative result could suggest that the ala gene cluster is not expressed in this system, that an additional enzyme(s) outside of the gene cluster might be required for L-alanosine production, or that *S. alanosinicus* produces cofactors and chaperones that are necessary for L-alanosine production that are not present in *S. lividans* TK64.

We have also demonstrated the importance of the free-standing NRPS biosynthetic machinery to this pathway, both by generating genetic knockouts of *S. alanosinicus* and through *in vitro* biochemical assays. We have confirmed the role of L-Dap as a biosynthetic precursor by showing that AlaB is a functional homolog of the L-Dap synthase SbnB and characterizing a new L-Dap specific A domain. The previously characterized biosynthetic uses of L-Dap have been as a building block for NRPS assembly lines, so its utilization as the core of a secondary metabolite is unusual. This may suggest that as yet undiscovered non-ribosomal peptide natural products contain L-alanosine building blocks in the same manner as gramibactin contains L-graminine building blocks.[7] We have also demonstrated a critical role for NO_2^- synthesis in this pathway, having confirmed through stable-isotope feeding studies that L-aspartic acid is the source of the *N*-nitroso group and shown that AlaI and AlaJ generate NO_2^- from L-aspartic acid *in vitro*. The presence of known NO_2^-

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

generation enzymes in the *ala* gene cluster, and confirmation of their necessity for the production of **5**, provides preliminary insights into diazeniumdiolate formation.

However, the mechanism of diazeniumdiolate formation in L-alanosine biosynthesis remains to be determined, and the remaining proteins encoded by the *ala* gene cluster may prove to be important in this process. The hypothetical proteins AlaE and AlaG contain a GAF and PAS domain, respectively, and may be part of a nitric oxide sensing system. It is possible that the PAS domain, which contains residues for heme-binding, may be acting to bind nitric oxide (or nitrite) and promote N-N bond formation in L-alanosine biosynthesis. As highlighted earlier, it is also possible that an N-N bond forming enzyme(s) is encoded elsewhere in the *S. alanosinicus* genome. We have found a potential homolog of the diazotizing enzyme from cremeomycin biosynthesis[5] (CreM 53.9% identity, 67.0% similarity) in the *S. alanosinicus* genome, and while it appears to be part of a separate biosynthetic gene cluster, we have not yet ruled out its invovlement in L-alanosine biosynthesis. If this enzyme is not involved, the lack of known homologs of *N*–nitrosating enzymes in the *ala* gene cluster and the *S. alanosinicus* genome hints at a potentially distinct mechanism of N-N bond-formation in L-alanosine biosynthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. L. Zha for assistance with the ATP-PPi exchange assay, Dr. B. A. Schneider for assistance with the BODIPY-CoA loading assay, and Dr. A. J. Waldman for guidance with the nitrite production assay. M.E.M. acknowledges the Damon Runyon Foundation for a Postdoctoral Fellowship (DRG 2307-17). We acknowledge financial support from the NIH (DP2 GM105434), a Cottrell Scholar Award (to E.P.B.), a Camille Dreyfus Teacher-Scholar Award (to E.P.B.), and Harvard University.

References

[1]. Lundberg JO, Weitzberg E, Gladwin MT, Nat. Rev. Drug Discov. 2008, 7, 156–167. [PubMed: 18167491]

[2]. Lundberg JO, Weitzberg E, Cole JA, Benjamin N, Nat. Rev. Microbiol.2004, 2, 593–602. [PubMed: 15197394]

[3]. Weiss RB, Issell BF, Cancer Treat. Rev. 1982, 9, 313–330. [PubMed: 6762924]

[4]. (a) Ng TL, Rohac R, Mitchell AJ, Boal AK, Balskus EP, Nature 2019, 566, 94–99. [PubMed: 30728519] (b) He HY, Henderson AC, Du YL, Ryan KS, J. Am. Chem. Soc. 2019, 141, 4026–4033. [PubMed: 30763082]

[5]. (a) Waldman AJ, Pechersky Y, Wang P, Wang JX, Balskus EP, ChemBioChem 2015, 16, 2172–2175. [PubMed: 26278892] (b) Sugai Y, Katsuyama Y, Ohnishi Y, Nat. Chem. Biol. 2015, 12, 73–77. [PubMed: 26689788]

[6]. Jenul C, Sieber S, Daeppen C, Mathew A, Lardi M, Pessi G, Hoepfner D, Neuburger M, Linden A, Gademann K, Eberl L, Nat. Commun. 2018, 9, 1297. [PubMed: 29602945]

[7]. Hermenau R, Ishida K, Gama S, Hoffman B, Pfeifer-Leeg M, Plass W, Mohr JF, Wichard T, Saluz H-P, Hertweck C, Nat. Chem. Biol. 2018, 14, 841–843. [PubMed: 30061716]

[8]. Murthy YKS, Thiemann JE, Coronelli C, Sensi P, Nature 1966, 211, 1198–1199. [PubMed: 5970033]

[9]. Powis G, Kovach JS, Biochemical Pharmacology 1981, 30, 771–776. [PubMed: 7247961]

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

[10]. Alston TA, Bright HJ, Biochem. Biophys. Res. Commun. 1982, 105, 560–566. [PubMed: 7092872]

[11]. Kindler HL, Burris HA 3rd, Sandler AB, Oliff IA, Invest. New Drugs 2009, 27, 75–81. [PubMed: 18618081]

[12]. Hermenau R, Mehl JL, Ishida K, Dose B, Pidot SJ, Stinear TP, Hertweck C, Angew. Chem. Int. Ed. Engl 2019, 58, 13024–13029. [PubMed: 31276269]

[13]. Felnagle EA, Rondon MR, Berti AD, Crosby HA, Thomas MG, Appl. Environ. Microbiol. 2007, 73, 4162–4170. [PubMed: 17496129]

[14]. Thomas MG, Chan YA, Ozanic SG, Antimicrob. Agents Chemother. 2003, 47, 2823–2830. [PubMed: 12936980]

[15]. Dale SE, Doherty-Kirby A, Lajoie G, Heinrichs DE, Infect. Immun. 2004, 72, 29–37. [PubMed: 14688077]

[16]. Kobylarz MJ, Grigg JC, Takayama SJ, Rai DK, Heinrichs DE, Murphy ME, Chem Biol. 2014, 21, 379–388. [PubMed: 24485762]

[17]. Du Y-L, He H-Y, Higgins MA, Ryan KS, Nat. Chem. Biol2017, 13, 836–838. [PubMed: 28628093]

[18]. (a) Matsuda K, Hasebe F, Shiwa Y, Kanesaki Y, Tomita T, Yoshikawa H, Shin-ya K, Kuzuyama T, Nishiyama M, ACS Chem. Biol. 2017, 12, 124–131. [PubMed: 28103675] (b) Matsuda K, Tomita T, Shin-ya K, Wakimoto T, Kuzuyama T, Nishiyama M, J. Am. Chem. Soc. 2018, 140, 9083–9086. [PubMed: 30001119]

[19]. Twigg FF, Cai W, Huang W, Liu J, Sato M, Perez TJ, Geng J, Dror MJ, Montanez I, Tong TL, Lee H, Zhang W, ChemBioChem 2019, 20, 1145–1149. [PubMed: 30589194]

[20]. Wang KKA, Ng TL, Wang P, Huang ZD, Balskus EP, van der Donk WA, Nat. Commun. 2018, 9, 3687. [PubMed: 30206228]

[21]. Graham DE, Spain JC, Parry RJ, Hettich RL, Mahan KM, Klingeman DM, Giannone RJ, Gulvick CA, Fida TT. Nitration Enzyme Toolkit for the Biosynthesis of Energetic Materials. 2018, Oak Ridge National Lab, Oak Ridge, TN.

[22]. Xu Z, Sun Z, Li S, Xu Z, Cao C, Xu Z, Feng X, Xu H, Scientific Reports 2015, 5, 17400. [PubMed: 26632244]

[23]. (a) Zhao C, Song C, Luo Y, Yu Z, Sun M, FEBS Letters 2008, 582, 3125–3131. [PubMed: 18692050] (b) Kevany BM, Rasko DA, Thomas MG, Appl. Environ. Microbiol. 2008, 75, 1144–1155. [PubMed: 19098220]

[24]. Felnagle EA, Rondon MR, Berti AD, Crosby HA, Thomas MG, Appl. Environ. Microbiol. 2007, 73, 4162–4170. [PubMed: 17496129]

[25]. Hur GH, Vickery CR, Burkart MD, Nat. Prod. Rep. 2012, 29, 1074–1098 [PubMed: 22802156]

[26]. Chevrette MG, Aicheler F, Kohlbacher O, Currie CR, Medema MH, Bioinformatics 2017, 33, 3203–3210.

[27]. Bachman BO, Ravel J, 2009 Methods in enzymology, 458, 181–217. [PubMed: 19374984]

[28]. Nakamura H, Wang JX, Balskus EP, Chem. Sci. 2015, 6, 3816–3822. [PubMed: 29218151]

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

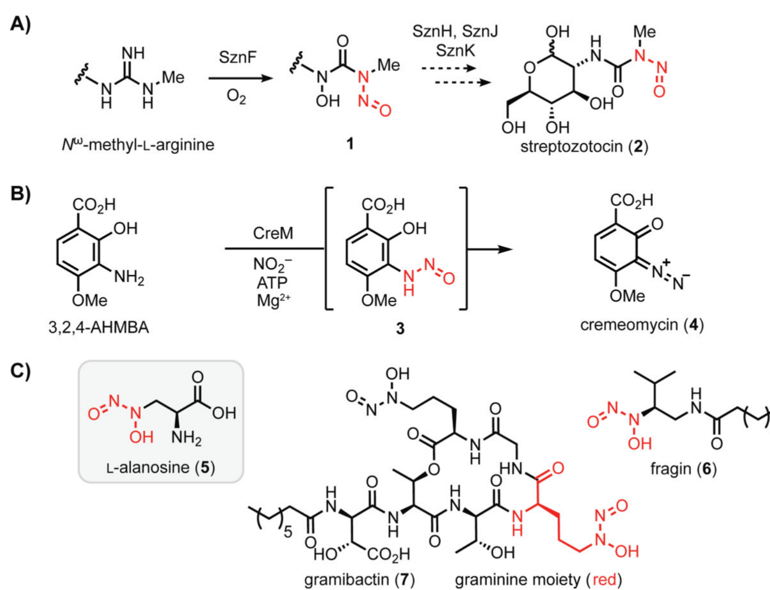


Figure 1.

Oxidized nitrogen species in the biosynthesis of N-N bond-containing natural products. A) Biosynthesis of the *N*-nitrosourea group in streptozotocin. B) Biosynthesis of cremeomycin may proceed through an *N*-nitrosamine intermediate. C) Selected diazeniumdiolate-containing natural products.

Author Manuscript

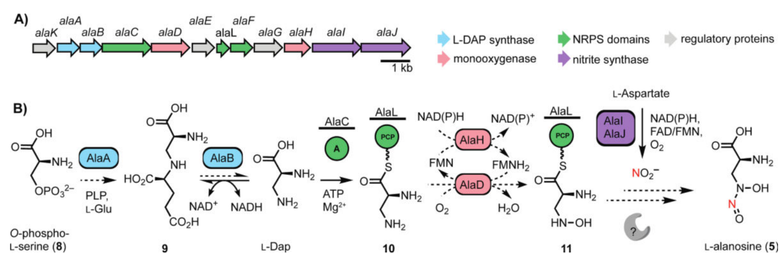


Figure 2.

The L-alanosine gene cluster encodes a pathway for diazeniumdiolate biosynthesis. A) The putative L-alanosine (*ala*) gene cluster and B) biosynthetic hypothesis for the production of **5** from *O*-phospho-L-serine (**8**). Solid arrows represent enzyme reactions confirmed *in vitro*, dashed arrows represent proposed transformations.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

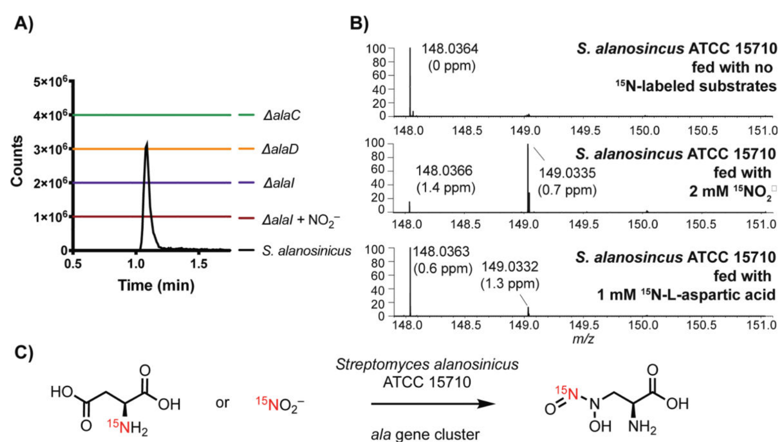


Figure 3.

Gene inactivation and feeding studies link *ala* biosynthetic genes to L-alanosine (**5**) production A) Extracted ion chromatograms (EICs) of **5** ($[M-H]^-$ = 148.0364) in fermentation extracts of gene deletion mutants and wild type *S. alanosinicus*. The EICs are generated within a 5 ppm window. B) Feeding studies using ^{15}N sources with wild type *S. alanosinicus* to determine the source of the distal *N*-nitroso nitrogen atom of **5**. LC-MS/MS analysis confirmed the distal *N*-nitroso nitrogen was labeled when ^{15}N-nitrite was fed (Figure S2 and S3). Incorporation of ^{15}N into **5** was also observed when 3 mM $^{15}NO_2^-$ was fed, although the cell viability was lower due to toxicity (Figure S3) C) Summary of the results of the feeding studies.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

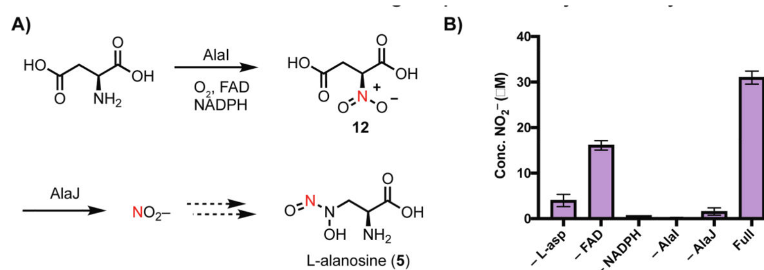


Figure 4.

AlaI and AlaJ are a nitrite synthase. A) Proposed mechanism of NO_2^- generation from L-aspartic acid by flavin dependent monooxygenase AlaI and nitrosuccinate lyase AlaJ B) AlaI and AlaJ produce NO_2^- from L-aspartic acid *in vitro.* NO_2^- production was detected by the Griess assay. Assay mixtures contained FAD (10 μM), NADPH (5 mM), N-Strep-AlaJ (5 μM), N-His_6-AlaI (5 μM) and L-aspartic acid (1 mM) in reaction buffer (50 mM HEPES, 10 mM $MgCl_2$, pH = 8.0) and were analyzed after a 1 h incubation at room temperature. AlaI purified with FAD, hence we still observed activity without exogenous FAD added. Data are mean ± standard deviation (s.d.) of two biological replicates.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

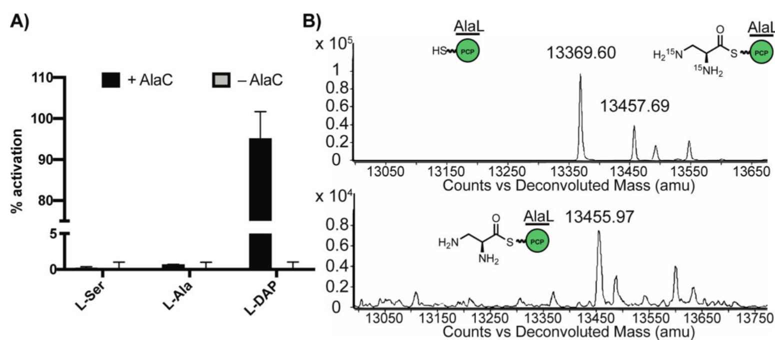


Figure 5.

AlaC selectively activates L-Dap for loading onto AlaL. A) ATP-$^{32}PP_i$ exchange assay shows that AlaC preferentially activates L-Dap over other amino acids from 100 μL incubations of N-His_6-AlaC (1 μM) with 5 mM dithiothreitol, 5 mM ATP, 1 mM of amino acid substrate, and 4 mM Na_4PPi/[^{32}P]PPi in reaction buffer (50 mM HEPES, 200 mM NaCl, 10 mM $MgCl_2$, pH = 8) at room temperature for 30 min. Data are mean ± s.d. of two biological replicates. B) Deconvoluted whole protein mass spectra (ESI+) showing holo-N-His_6-AlaL-C-His_6 (13369.60) is loaded with $^{15}N_2$-L-Dap (13457.69) or L-Dap (13455.97). 50 μL reaction mixtures were set up with coenzyme A (1 mM), Sfp (5 μM), and N-His_6-AlaL-C-His_6 (20 μM) in a solution of reaction buffer (50 mM HEPES, 200 mM NaCl, 10 mM $MgCl_2$, pH = 8). After incubation at room temperature for 2 h, AlaC (20 μM) and L-Dap or L-$^{15}N_2$-Dap (250 μM) were added, followed by ATP (5 mM) to initiate the reaction. Incubated for 1 h at room temperature.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 1.

Annotations of the alanosine (*ala*) biosynthetic gene cluster. The nucleotide sequences have been deposited into the NCBI database (Genbank accession numbers for AlaA-L are MN603934-MN603945).

Protein	Size (aa)	Predicted Function	Closest Homolog	Accession #	%Identity/ %Similarity
AlaA	324	2,3-diaminopropionate biosynthesis protein	SbnA, *Streptomyces hirsutus*	WP_055594931.1	87/91
AlaB	353	2,3-diaminopropionate biosynthesis protein	SbnB, *Streptomyces hirsutus*	WP_055594930.1	84/89
AlaC	557	amino acid adenylation domain-containing protein	*Streptomyces hirsutus*	WP_055594929.1	79/84
AlaD	385	acyl-CoA dehydrogenase	*Streptomyces hirsutus*	WP_055594947.1	89/94
AlaE	190	hypothetical protein	*Streptomyces hirsutus*	WP_055594928.1	80/88
AlaF	237	thioesterase	hypothetical protein, *Streptomyces hirsutus*	WP_079034952.1	76/83
AlaG	286	hypothetical protein	*Streptomyces hirsutus*	WP_055594925.1	86/92
AlaH	177	NADPH-dependent FMN reductase	*Streptomyces hirsutus*	WP_055594946.1	87/92
AlaI	657	FAD/NAD(P)-binding protein	FAD/NAD(P)-binding protein [*Streptomyces kanamyceticus*]	WP_055544225.1	76/82
AlaJ	470	nitro-succinate lyase	3-carboxy-*cis,cis*-muconate cycloisomerase [*Streptomyces sp. BK161*]	TDT22211.1	75/80
AlaK	273	SARP family regulator	CreF [*Streptomyces cremeus*]	ALA99205.1	57/69
AlaL	79	carrier protein	MULTISPECIES: [*Streptomyces*]	WP_030834819.1	91/79