

SCIENTIFIC REPORTS



OPEN

Towards Biocontained Cell Factories: An Evolutionarily Adapted *Escherichia coli* Strain Produces a New-to-nature Bioactive Lantibiotic Containing Thienopyrrole-Alanine

Anja Kuthning¹, Patrick Durkin², Stefan Oehm², Michael G. Hoesl², Nediljko Budisa² & Roderich D. Süßmuth¹

Received: 15 June 2016
Accepted: 25 August 2016
Published: 16 September 2016

Genetic code engineering that enables reassignment of genetic codons to non-canonical amino acids (ncAAs) is a powerful strategy for enhancing ribosomally synthesized peptides and proteins with functions not commonly found in Nature. Here we report the expression of a ribosomally synthesized and post-translationally modified peptide (RiPP), the 32-mer lantibiotic lichenicidin with a canonical tryptophan (Trp) residue replaced by the ncAA L- β -(thieno[3,2-*b*]pyrrolyl)alanine ([3,2]Tpa) which does not sustain cell growth in the culture. We have demonstrated that cellular toxicity of [3,2]Tpa for the production of the new-to-nature bioactive congener of lichenicidin in the host *Escherichia coli* can be alleviated by using an evolutionarily adapted host strain MT21 which not only tolerates [3,2]Tpa but also uses it as a proteome-wide synthetic building block. This work underscores the feasibility of the biocontainment concept and establishes a general framework for design and large scale production of RiPPs with evolutionarily adapted host strains.

In the frame of our efforts to generate prototype biocontained strains exhibiting genetic and trophic isolation and expanded biological functions^{1,2} we aimed to expand our previous attempts to engineer ribosomally synthesized and post-translational modified peptides (RiPPs) by ribosomally introducing ncAAs into their sequences. Thereby, we are pursuing Xenobiology with the aim to implement various man-made chemical syntheses in living cells. Whereas Synthetic Biology mainly works with naturally occurring building blocks and a canonical chemistry, Xenobiology uses non-natural building blocks and non-canonical chemistries³.

Currently, the development of alternative biological systems with radically altered genetic codes implies massive genome engineering⁴. However, approaches aiming at the generation of cell factories as platforms are still immature, as they generally suffer from synthetic lethal mutations, codon reversions and dramatically decreased fitness during the genome assembly process⁵. On the other hand, widely used orthogonal pairs are not as active and accurate as natural aminoacyl-tRNA synthetases with related cognate tRNAs⁶. Our alternative strategy for experimental genetic code evolution towards changes in its biochemistry and to achieve biocontainment relies on the global substitution of canonical amino acids with ncAAs assisted with simple metabolic engineering^{7,8}. Recently, we described a long-term evolution experiment which led to the reassignment of all 20,899 Trp codons in the genetic code of the bacterium *Escherichia coli*². Cultivation of the *E. coli* strain in defined synthetic media resulted in the generation of the bacterial strain MT21, which is capable of proteome-wide Trp \rightarrow [3,2]Tpa substitutions in response to all TGG codons in the genome. These evolved bacteria with their new-to-nature amino

¹Technische Universität Berlin, Institut für Chemie, Biologische Chemie, Straße des 17. Juni 124, Berlin, 10623, Germany. ²Technische Universität Berlin, Institut für Chemie, Biokatalyse, Müller-Breslau-Straße 10, Berlin, 10623, Germany. Correspondence and requests for materials should be addressed to N.B. (email: budisa@biocat.tu-berlin.de) or R.D.S. (email: suessmuth@chem.tu-berlin.de)

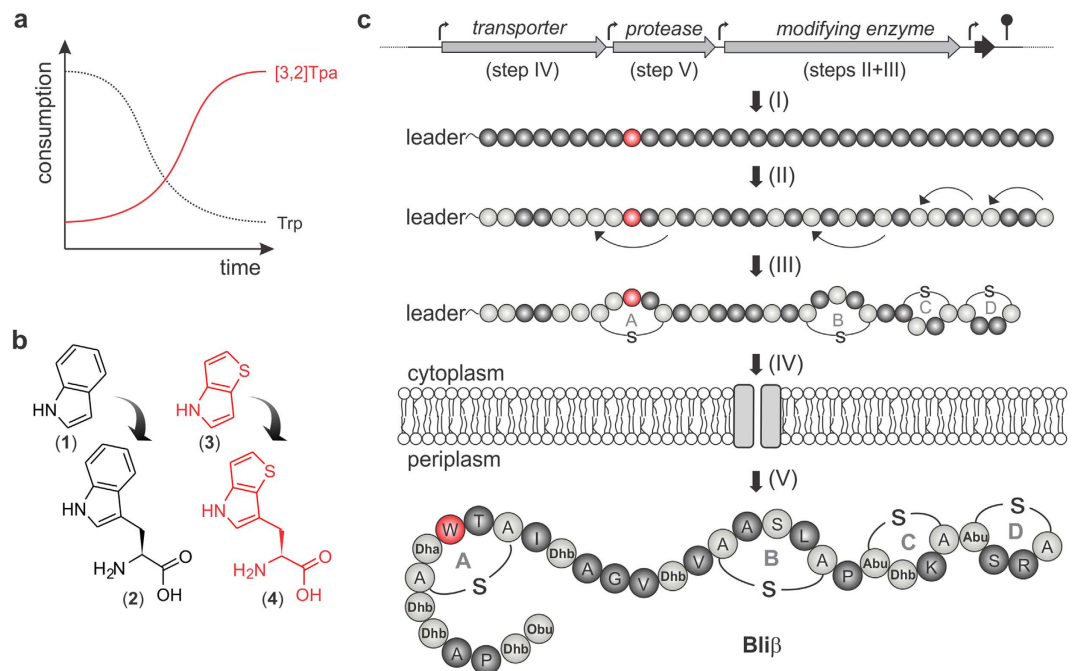


Figure 1. Strategy and prerequisites for the production of congeneric, ribosomally synthesized peptides in emancipated *E. coli* cells. (a) Evolutionarily adapted *E. coli* cells are cultivated in defined minimal medium until residual Trp is consumed and cells solely grow on [3,2]Tpa. (b) The Trp (2) progenitor indole (1) is replaced by 4*H*-thieno[3,2-*b*]pyrrole (Tp) (3), which in turn is converted into [3,2]Tpa (4) by the tryptophan synthetase. (c) Scheme of the biosynthesis of Bli β . The linear precursor peptide is translationally synthesized from the corresponding gene (indicated in black) (I). Dehydrations and thioether bridges are enzymatically installed (II–III) (residues depicted in light grey) and the modified peptide is exported via a specific transporter (IV). Extracellularly, a protease activates the peptide by removal of an N-terminal hexapeptide (V). Dhb, didehydrobutyryl; Dha, didehydroalanine; Obu, 2-oxobutyryl; Abu, aminobutyrate.

acid composition are capable of robust growth in the complete absence of the canonical (natural) amino acid Trp (Fig. 1a,b)^{2,9}. Previously, we and others have applied various methods, aiming to engineer RiPPs by ribosomally introducing ncAAs into their sequences *in vitro* and *in vivo*, exploiting the natural biosynthetic pathways^{1,10–12}. Nevertheless, supplementation-based incorporation (SPI) only allows for the insertion of isosteric analogues of cAAs, the structural diversity of which is restricted by the promiscuity of the respective tRNA and aminoacyl-tRNA synthetase and limited by the use of auxotrophic strains^{13–15}. Expanding the structural complexity of the ncAA regardless of the amino acid to be replaced, can be achieved by stop-codon-suppression (SCS) or reassignment of a sense codon but requires the design of new pairs of orthogonal tRNA and the corresponding aminoacyl-tRNA synthetases^{8,16–22} and genetic modifications such as introduction of the respective codon in the addressed gene and removing of suppressor tRNAs or release factor 1 for improved yields^{23–26}. Herein we report the use of fully adapted *E. coli* MT21 as a platform for production of ncAAs-containing small-molecule-type antibiotic peptides, which undergo massive post-translational modifications, being only recently addressed in the frame of single protein/peptide recombinant production by using standard expression strains^{1,10,11}. The transfer of xenobiological concepts and ideas to peptides with antibiotic properties opens up a new structural space for various compound classes and thus possibly altered or enhanced bioactivities. Peptide natural products, which are ribosomally synthesized and post-translationally modified peptides (RiPPs) comprise of various subgroups, e.g. lanthipeptides^{27–30}, microviridins^{31,32}, lasso peptides³³, or linear azole containing peptides^{34,35} with various characteristic structural features³⁶. We apply the assembly of the otherwise toxic amino acid L- β -(thieno[3,2-*b*]pyrrolyl) alanine ([3,2]Tpa)³⁷ (Fig. 1b) to an evolved *E. coli* strain which carries the gene cluster for the heterologous production of the congeneric lantibiotic lichenicidin. Lichenicidin is a two-component lantibiotic originating from *Bacillus licheniformis*³⁸. The two peptides, Bli α and Bli β , are assumed to act synergistically on the cell wall of Gram-positive bacteria in a manner that has been similarly described for other two-component lantibiotics^{39–42}. In this scenario, the α -peptide binds to the peptidoglycan precursor lipid II, and the β -peptide is subsequently recruited to the cell wall to induce pore formation^{43–45}. The lichenicidin gene cluster (*lic* cluster, 15 kb) comprises of 14 genes (see Supplementary Fig. S1)⁴⁶, of which only six are essential for heterologous expression of the lichenicidin peptides (Bli α and Bli β) in *E. coli*⁴⁷. Production of Bli α and Bli β includes a number of biosynthetic steps (Fig. 1c): subsequent to the ribosomal biosynthesis, an intramolecular crosslinking occurs between dehydrated Ser or Thr and Cys residues to form the diamino diacid lanthionine (Lan) or methylanthionine (MeLan), respectively. These modifications provide structural stability and rigidity, making lanthipeptides particularly attractive compounds as potential novel antibiotics⁴⁸. The *licA1* and *licA2* structural genes each code for the 72-mer linear precursor peptide of Bli α and Bli β , respectively. Two sequence-specific modifying enzymes interact with the

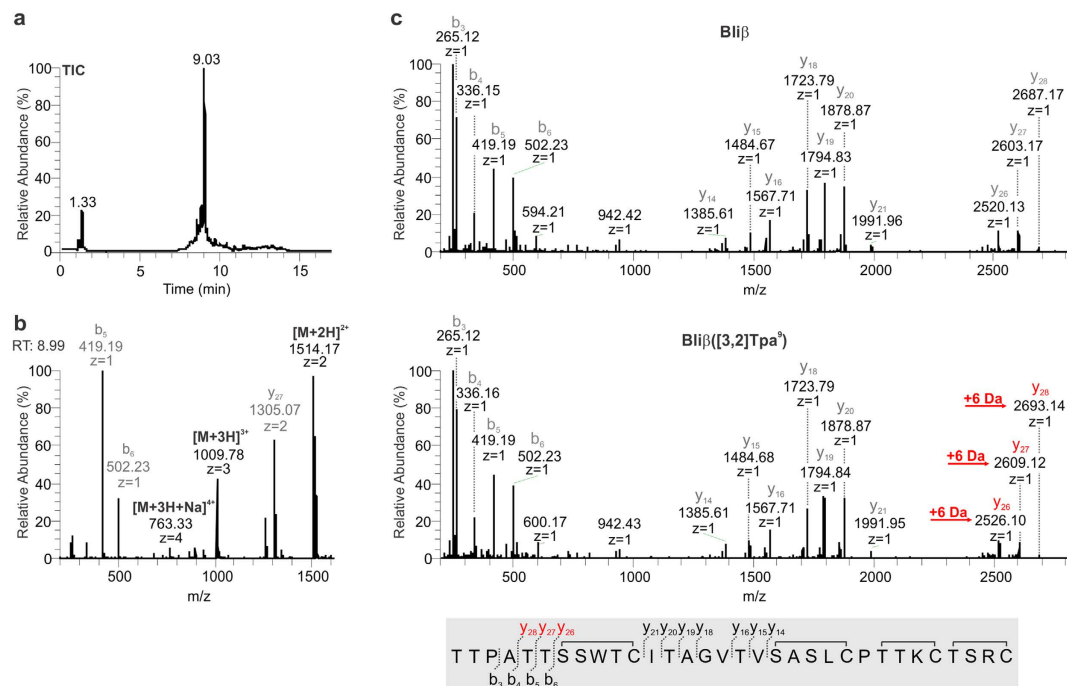


Figure 2. LC-ESI-MS analytics of congeneric Bliβ([3,2]Tpa⁹). (a) Total ion chromatogram of Bliβ ([3,2]Tpa⁹) extracted with *n*-butanol. (b) Mass spectrum of Bliβ([3,2]Tpa⁹) ($[M_{\text{calc}} + 2H]^{2+} = 1514.17$, $[M_{\text{calc}} + 3H]^{3+} = 1009.78$) with annotated fragment masses. (c) HR-ESI-MS² analysis of wild type Bliβ ($[M + 2H]^{2+} = 1511.18$ Da) and congeneric Bliβ([3,2]Tpa⁹) ($[M + 2H]^{2+} = 1514.17$ Da). Characteristic mass shifts of 6 Da due to incorporation of [3,2]Tpa as a surrogate for Trp are indicated in red color.

leader sequence in the corresponding precursor peptide and catalyze the thioether formation in the core region of the respective peptides⁴⁶. A specific membrane transporter protein, carrying a peptidase domain, removes a large portion of the leader sequence prior to the export of the peptide from the cell. An N-terminal hexapeptide remains covalently bound to the modified β-peptide and is not removed until the peptide is translocated outside of the cell, keeping the peptide inactive during the transport. An extracellular protease cleaves off the remaining part of the leader peptide and releases the active peptide (Fig. 1c)⁴⁶.

For the assembly of the Trp-congener [3,2]Tpa (4) we chose the β-peptide of lichenicidin, because it naturally carries one Trp in position 9 of the core peptide (see Supplementary Fig. S2). Another advantage is that it is a genetically manageable RiPP system, which can be applied in the heterologous *E. coli* host⁴⁹. According to our approach, by cultivating the evolutionarily adapted strain *E. coli* MT21(DE3) in minimal medium containing a defined set of amino acids with 4*H*-thieno[3,2-*b*]pyrrole (Tp) (3) replacing indole (1) (Fig. 1b) will increase the selective pressure in favor of translational incorporation of the Trp analogue over Trp into the protein (Fig. 1a). The challenging aspect of our approach is that all of the previously described biosynthetic steps must be able to incorporate [3,2]Tpa globally into all biosynthesized proteins, including those of the post-translational machinery.

Results

Cells of strain *E. coli* MT21(DE3) were transformed with the plasmid pRSFDuet-1_TPM2A2 (see Supplementary Fig. S1), which carries the required genes for Bliβ production in *E. coli*⁴⁹. The resulting strain *E. coli* MT21.1 HPβ was used to express the congeneric Bliβ carrying [3,2]Tpa. The cells were first cultivated in LB medium as a starter culture and subsequently washed and cultivated in minimal medium containing Tp as a precursor for [3,2]Tpa synthesis, until the remaining Trp was consumed (Fig. 1a). Taking the biosynthetic pathway of Bliβ into consideration, we assumed that only the fully processed peptides are exported from the cell and we expected all active peptides to be exclusively located in the culture supernatant. Consequently, the peptides were extracted from the supernatant by addition of *n*-butanol. Indeed, we detected the doubly ($[M + 2H]^{2+} = 1514.17$), triply ($[M + 3H]^{3+} = 1009.78$) and quadruply ($[M + 3H + Na]^{4+} = 763.33$) charged molecular masses of the congeneric peptide by HPLC-MS (Fig. 2a,b). In order to verify the incorporation of [3,2]Tpa into Bliβ, we additionally performed MS/MS experiments, which confirmed the specific mass shift of 6 Da (indole $[M_{\text{calc}} = 117.06$ Da] → 4*H*-thieno[3,2-*b*]pyrrole $[M_{\text{calc}} = 123.01$ Da]) in the A-ring of Bliβ, thus replacing Trp in the peptide (Fig. 2c). In order to assess the specificity, efficiency and the robustness of the expression system we again analyzed the supernatant extracts by means of ESI-MS. When the adapted cells were cultivated in minimal medium with indole as source for Trp synthesis, wild type Bliβ was produced (Fig. 3a). If both, indole and Tp are present in the culture medium, indole is preferably converted into Trp and used for ribosomal synthesis of the peptides (data not shown). When the adapted cells were cultivated in minimal medium supplemented with Tp, the exclusive production of congeneric Bliβ([3,2]Tpa⁹) was observed (Fig. 3b), which exemplifies the robustness

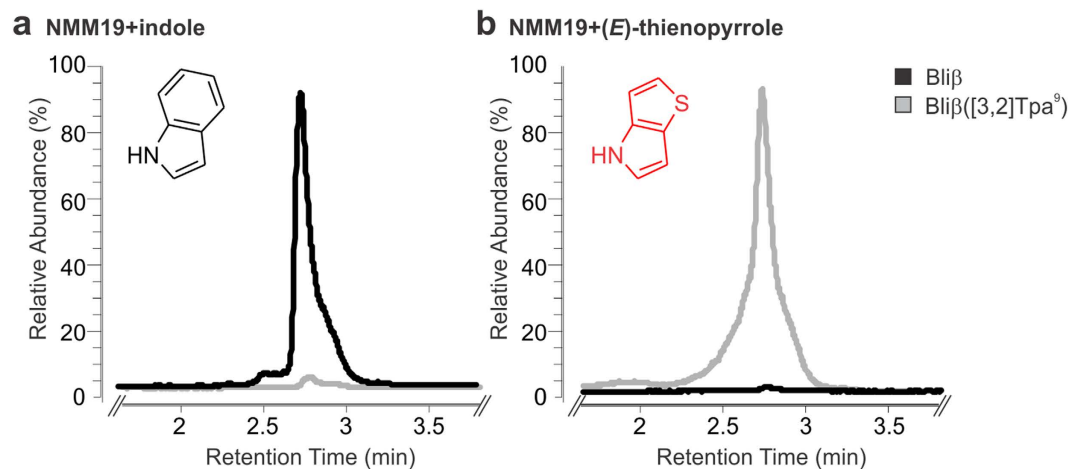


Figure 3. Relative abundance of wild type Bli β compared to congeneric Bli β ([3,2]Tpa 9). *E. coli* MT21(DE3) cells were cultivated in NMM19 medium supplemented with (a) indole and (b) 4H-thieno[3,2-*b*]pyrrole (Tp). Peptides were quantified by HPLC-ESI-MS analysis.

of the expression system by not allowing the production of the wild type Bli β . To assess the bioactivity of this new-to-nature compound, the concentration was determined by mass spectrometric analysis (see Supporting Information). Dried extracts from a cultivation of the same strain in a medium supplemented with indole, instead of Tp, contained wild type Bli β . We measured the amount of Bli β proportional to the amount of Bli β ([3,2]Tpa 9) produced by the strain cultivated in NMM19 + Tp and NMM19 + indole, respectively and observed a 2-fold decrease in production of the congener compared to the wild type (data not shown). In general the peptide yields were much lower than that previously reported⁴⁹, which can be attributed to the limitations of the non-optimal culture medium (NMM19) and genetic modifications necessary for this experimental setup. Considering the differences in the production of Bli β peptides, we adjusted the amounts of Bli β and Bli β ([3,2]Tpa 9) to 0.5 μ M and used both in an antimicrobial agar diffusion assay against *Micrococcus luteus* (Fig. 4). As expected, the separate testing of the wild type peptides Bli α and Bli β did not show any antibacterial effect, while the combination of both peptides resulted in a clear halo indicating antimicrobial bioactivity. Interestingly, the congeneric peptide Bli β ([3,2]Tpa 9) did not show a decrease in bioactivity, suggesting that the introduction of [3,2]Tpa does not influence the overall structure of the peptide, nor does it negatively affect the interaction with Bli α .

In this study, we firmly prove our working hypothesis, that the application of adapted strains is not only suitable for the expression of a one single protein but also encompasses the possibility for the production of new-to-nature bioactive secondary metabolites, which are synthesized via complex biosynthetic pathways, requiring a relaxed substrate specificity of the PTM machinery for the altered peptide sequence. Moreover, we could demonstrate and confirm the versatile applicability of the complex biosynthesis of lichenicidin, that involves the interaction and catalytic reactions of several proteins, with regard to the exchange of an amino acid with a particular surrogate, beyond techniques aiming at amino acid exchange that have been addressed so far.

Discussion

Reprogrammed cells or proteins equipped with synthetic structures are currently usually considered as useful tools for academic research or small applications. However, this engineering can even have practical importance when applications such as bioremediation (in open systems) biocatalysts or peptide-based drugs (closed systems) are considered⁵⁰. Furthermore, the incorporation of various ncAAs into the proteome⁵¹ or in some *E. coli* essential genes^{4,5} can be envisioned as a promising biosafety approach: as long as the ncAAs is absent from the medium, no bacterial growth is possible. This is an important prerequisite for biocontainment which is still not fully achieved in our MT21 strain. Namely, it should be noted that 20,899 TGG codons are only trophically reassigned (i.e. the meaning of a codon is redefined throughout the whole translational machinery for the evolved cells only in the defined synthetic medium). That means the supplementation of cells in such a medium with the canonical substrate Trp reverses them to 'natural' ones as they still favor the incorporation of the canonical building block. To achieve a nutrient-independent reassignment (i.e. 'real' codon reassignment) for the all genome TGG codons in *E. coli* – an experimental strategy for biocontainment needs to be developed and executed.

Nonetheless, for the first time we have provided a solid proof-of-principle for the application of an evolutionarily adapted *E. coli* strain in production of new-to-nature modified lantibiotics. For future bioengineering purposes, our system and its improved versions will doubtlessly provide a manifold of opportunities to design various novel ribosomally synthesized compounds. State-of-the-art modified lantipeptides are produced (semi-) synthetically^{52–55}, and currently are limited to only a few applications in a healthcare setting^{56,57}. However, with our methodology we could open up the opportunity to incorporate non-canonical amino acids, enabling us to push forward the *in vivo* diversification of difficult-to-synthesize RiPPs. Recent reports on the development of super-pathogens⁵⁸ emphasize the unabated need for new antibiotics, which can circumvent naturally arising host defense mechanisms^{59,60}. Hence, the engineering of lantibiotics with chemical structures, rarely occurring in Nature, is a necessary approach to fill the void in developing new antimicrobial compounds⁶¹.

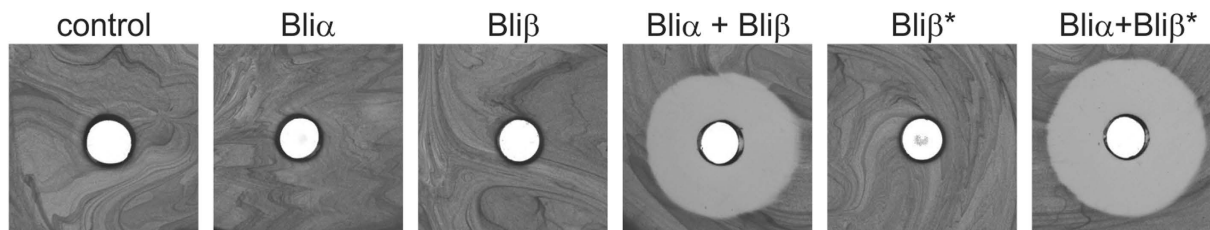


Figure 4. Antimicrobial activity of lichenicidin peptides. Bli α , Bli β and Bli β ([3,2]Tpa⁹), indicated as Bli β *, were tested separately (concentration 0.5 μ M) and in equimolar (1:1) combinations against the indicator strain *Micrococcus luteus* (DSM-1790). The assay was performed in triplicate.

Methods

Cloning. The plasmid pSTB7, carrying the *trpBA* gene originating from *Salmonella typhimurium* which is required for conversion of indole into tryptophan was described earlier². Additionally, we used the vector pRSFDuet-1_ TPM2A2 which carries four genes required for Bli β production in *E. coli*⁴⁹. For compatibility reasons we exchanged the kanamycin resistance gene of pRSFDuet-1_ TPM2A2 (See Supplementary Fig. S1) for an ampicillin (*amp*) resistance by heterologous recombination applying the arabinose-inducible λ -recombinase system (a kind gift from Dr. Bertolt Gust, Tübingen)⁶². The *ampR* gene was amplified from pET-Duet-1 (Novagen) using the primers AK163 (5'-TTCAAATATGTATCCGCTCATGAGACAATAACCC-3') and AK164 (5'-TGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAATTAATTCTTACCAATGCTTAATCAGTGAGGCACC-3').

Strains. The initial strain used for evolutionary adaptation to the non-natural amino acid Tpa was *E. coli* K12 W3110 (CGSC#7679). The generation of thus Trp emancipated strain has been published earlier² and will only be summarized in brief: the genes for the Trp biosynthesis pathway were deleted (Δ *trpLEDCBA*) and substituted by *trpBA* on an extrachromosomal plasmid pSTB7. Hence, Trp-synthetase, the gene product of *trpBA*, enables the strain to convert indole into Trp, facilitating to feed the strain either with indole or indole analogues. Adaptation to the indole derivative 4*H*-thieno[3,2-*b*]pyrrole (Tp) finally gave rise to the strain *E. coli* MT21 which continuously could feed on this substrate. As the expression system for lichenicidin requires a T7-polymerase, cells were transformed with a λ DE3-lysogenization kit (Novagen, Merck Millipore). The resulting MT21 (DE3) cells were transformed with plasmid pRSFDuet-1_ TPM2A2(*amp*).

Culture Conditions. 500 μ L of an overnight culture in LB-medium were collected and washed twice in NMM19 medium (7.5 mL 1 M (NH₄)₂SO₄, 68 mL 1 M KH₂PO₄, 31 mL 1 M K₂HPO₄, 1.7 mL 5 M NaCl, 20 mL 1 M glucose, 1 mL 1 M MgSO₄, 1 mL Ca²⁺ (1 mg mL⁻¹), 1 mL Fe²⁺ (1 mg mL⁻¹), 1 mL trace elements, ad 1 L deionised H₂O, supplemented with 19 canonical amino acids solution, whereupon Trp has been substituted by 4*H*-thieno[3,2-*b*]pyrrole (Tp). Chemical synthesis of Tp has been described earlier². After the second wash the cells were used for inoculation of a 50 mL culture of NMM19 + [3,2]Tp (NMM19 medium supplemented with 0.1 mM of the indole surrogate Tp). The cultures were incubated at 37 °C, 200 rpm until they reached stationary phase. The procedure was repeated for another selection round. From the second 50 mL culture a total of 10 L of main expression culture was inoculated. The main cultures were incubated until optical density was 0.2 at OD₆₀₀. Gene expression was induced by addition of 0.5 mM IPTG (f.c.) and cultures were incubated at 30 °C, 160 rpm for 20 h. For production of wild type lichenicidin the strains *E. coli* HP α and *E. coli* HP β were cultivated as described earlier⁴⁹.

Peptide Extraction. Cultures were harvested by centrifugation and supernatant was collected as fully processed congeneric peptides were expected to be exported from the cell. 1/5 volume of *n*Bu was added to the supernatant and incubated shaking. Dried *n*Bu extracts were dissolved in 70% ACN and precipitated in ice-cold acetone for 16 h. Pure Bli α and Bli β were isolated as described earlier⁴⁹.

Mass Spectrometric Analysis and Quantification. LC-ESI-MS and LC-ESI-MS² experiments were performed on an ESI-LTQ-Orbitrap (Thermo Scientific). For chromatographic separation a Grom-Sil 120 ODS-5 ST (100 mm \times 2 mm, 5 μ m) column (GRACE) was used with an Agilent 1260 HPLC system. A gradient starting at 5% solvent B, increasing to 100% solvent B over 10 min, then held at 100% solvent B for 3 min, then over 0.1 min to 5% solvent B followed by 3.9 min isocratic at 5% solvent B was applied with a flow rate of 0.2 mL min⁻¹ (solvent A: H₂O + 0.1% HFO, solvent B: ACN + 0.1% HFO). MS² spectra were obtained from an IDA Top2 scan using HCD (CE = 35 eV). For quantification LC-ESI-MS/MS using multiple reaction monitoring (MRM) analytics were performed on an ESI-Triple-Quadrupole LC-MS 6460 with a preceding Agilent 1290 Infinity HPLC system (Agilent Technologies). A GRACE Grom-Sil 300 Octyl-6 MB (2 \times 50 mm, 3 μ m) column was applied for an acetonitrile gradient starting at 5% B, then increasing to 20% B in 0.5 min, then to 70% B in 4 min, and finally to 100% B in 0.2 min, followed by a 1.3 min isocratic hold on 100% B. The flowrate was 0.5 mL min⁻¹. For quantitation of lichenicidin peptide yields the [M + 3H]³⁺ adducts of the peptides were used as precursor ions. For MRM the mass transitions for Bli β m/z 1007.8 \rightarrow 1302.0, and m/z 1007.8 \rightarrow 265.1 and for Bli β ([3,2]Tpa⁹) m/z 1009.5 \rightarrow 1304.5, and m/z 1009.5 \rightarrow 265.1 were used. Peptide concentrations were compared to a standard curve from purified wildtype Bli β (see Supplementary Fig. S4).

Antibacterial Assay. Antibacterial activity was assessed in Mueller Hinton Broth Agar Plates (Difco) against *Micrococcus luteus* DSM-1790 at a final concentration of 0.02 OD₆₀₀. Supernatant extracts from cultures expressing Bli β or Bli β ([3,2]Tpa⁹) were analyzed by mass spectrometry on an ESI-Triple-Quadrupole with respect to compound concentration and compared to a standard curve. The respective compound was diluted to a final concentration of 0.5 μ M and mixed with equal amounts of purified Bli α in 70% ACN and applied to a 5 mm well on the plate. Inhibition zones were determined after 18 h incubation at 30 °C.

References

- Budisa, N. Expanded genetic code for the engineering of ribosomally synthesized and post-translationally modified peptide natural products (RiPPs). *Curr. Opin. Biotechnol.* **24**, 591–598 (2013).
- Hoesl, M. G. *et al.* Chemical evolution of a bacterial proteome. *Angew. Chem. Int. Ed.* **54**, 10030–10034 (2015).
- Acevedo-Rocha, C. G. & Budisa, N. On the road towards chemically modified organisms endowed with a genetic firewall. *Angew. Chem. Int. Ed.* **50**, 6960–6962 (2011).
- Mandell, D. J. *et al.* Biocontainment of genetically modified organisms by synthetic protein design. *Nature* **518**, 55–60 (2015).
- Rovner, A. J. *et al.* Recoded organisms engineered to depend on synthetic amino acids. *Nature* **518**, 89–93 (2015).
- Nehring, S., Budisa, N. & Wiltschi, B. Performance analysis of orthogonal pairs designed for an expanded eukaryotic genetic code. *PLoS One* **7**, e31992 (2012).
- Ma, Y., Biava, H., Contestabile, R., Budisa, N. & di Salvo, M. L. Coupling bioorthogonal chemistries with artificial metabolism: Intracellular biosynthesis of azidohomoalanine and its incorporation into recombinant proteins. *Molecules* **19**, 1004–1022 (2014).
- Bohlke, N. & Budisa, N. Sense codon emancipation for proteome-wide incorporation of noncanonical amino acids: Rare isoleucine codon AUA as a target for genetic code expansion. *FEMS Microbiol. Lett.* **351**, 133–144 (2014).
- Bacher, J. M. & Ellington, A. D. Selection and characterization of *Escherichia coli* variants capable of growth on an otherwise toxic tryptophan analogue. *J. Bacteriol.* **183**, 5414–5425 (2001).
- Oldach, F. *et al.* Congeneric lantibiotics from ribosomal *in vivo* peptide synthesis with noncanonical amino acids. *Angew. Chem. Int. Ed.* **51**, 415–418 (2012).
- Al Toma, R. S. *et al.* Site-directed and global incorporation of orthogonal and isostructural noncanonical amino acids into the ribosomal lasso peptide capistruiin. *ChemBioChem* **16**, 503–509 (2015).
- Luo, X. *et al.* Recombinant thiopeptides containing noncanonical amino acids. *Proc. Natl. Acad. Sci. USA* **113**, 3615–3620 (2016).
- Ross, J. B., Szabo, A. G. & Hogue, C. W. Enhancement of protein spectra with tryptophan analogs: Fluorescence spectroscopy of protein-protein and protein-nucleic acid interactions. *Methods Enzymol.* **278**, 151–190 (1997).
- Budisa, N. *et al.* Residue-specific bioincorporation of non-natural, biologically active amino acids into proteins as possible drug carriers: Structure and stability of the per-thiaproline mutant of annexin V. *Proc. Natl. Acad. Sci. USA* **95**, 455–459 (1998).
- Budisa, N. *et al.* Toward the experimental codon reassignment *in vivo*: Protein building with an expanded amino acid repertoire. *FASEB J.* **13**, 41–51 (1999).
- Fekner, T., Li, X., Lee, M. M. & Chan, M. K. A pyrrolysine analogue for protein click chemistry. *Angew. Chem. Int. Ed.* **48**, 1633–1635 (2009).
- Fekner, T. & Chan, M. K. The pyrrolysine translational machinery as a genetic-code expansion tool. *Curr. Opin. Chem. Biol.* **15**, 387–391 (2011).
- Anderson, J. C. & Schultz, P. G. Adaptation of an orthogonal archaeal leucyl-tRNA and synthetase pair for four-base, amber, and opal suppression. *Biochemistry* **42**, 9598–9608 (2003).
- Chatterjee, A., Xiao, H. & Schultz, P. G. Evolution of multiple, mutually orthogonal prolyl-tRNA synthetase/tRNA pairs for unnatural amino acid mutagenesis in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **109**, 14841–14846 (2012).
- Kwon, I., Wang, P. & Tirrell, D. A. Design of a bacterial host for site-specific incorporation of p-bromophenylalanine into recombinant proteins. *J. Am. Chem. Soc.* **128**, 11778–11783 (2006).
- Mukai, T. *et al.* Reassignment of a rare sense codon to a non-canonical amino acid in *Escherichia coli*. *Nucleic Acids Res.* **43**, 8111–8122 (2015).
- Reichert, A. J., Poxleitner, G., Dauner, M. & Skerra, A. Optimisation of a system for the co-translational incorporation of a keto amino acid and its application to a tumour-specific Anticalin. *Protein Eng. Des. Sel. PEDS* **28**, 553–565 (2015).
- Nilsson, M. & Rydén-Aulin, M. Glutamine is incorporated at the nonsense codons UAG and UAA in a suppressor-free *Escherichia coli* strain. *Biochim. Biophys. Acta* **1627**, 1–6 (2003).
- Krishnakumar, R. & Ling, J. Experimental challenges of sense codon reassignment: an innovative approach to genetic code expansion. *FEBS Lett.* **588**, 383–388 (2014).
- Mukai, T. *et al.* Codon reassignment in the *Escherichia coli* genetic code. *Nucleic Acids Res.* **38**, 8188–8195 (2010).
- Zengh, Y. *et al.* Performance of optimized noncanonical amino acid mutagenesis systems in the absence of release factor 1. *Mol. Biosyst.* **12**, 1746–1749 (2016).
- Meindl, K. *et al.* Labyrinthopeptins: A new class of carbacyclic lantibiotics. *Angew. Chem. Int. Ed.* **49**, 1151–1154 (2010).
- Müller, W. M., Schmiederer, T., Enslé, P. & Süßmuth, R. D. *In vitro* biosynthesis of the prepeptide of type-III lantibiotic labyrinthopeptin A2 including formation of a C-C bond as a post-translational modification. *Angew. Chem. Int. Ed.* **49**, 2436–2440 (2010).
- Völler, G. H. *et al.* Characterization of new class III lantibiotics—erythraeptin, avermipeptin and griseopeptin from *Saccharopolyspora erythraea*, *Streptomyces avermitilis* and *Streptomyces griseus* demonstrates stepwise N-terminal leader processing. *Chem Bio Chem* **13**, 1174–1183 (2012).
- Knerr, P. J. & van der Donk, W. A. Discovery, biosynthesis, and engineering of lantipeptides. *Annu. Rev. Biochem.* **81**, 479–505 (2012).
- Ishitsuka, M. O., Kusumi, T., Kakisawa, H., Kaya, K. & Watanabe, M. M. Microviridin. A novel tricyclic depsipeptide from the toxic cyanobacterium *Microcystis viridis*. *J. Am. Chem. Soc.* **112**, 8180–8182 (1990).
- Ziemert, N., Ishida, K., Liaimer, A., Hertweck, C. & Dittmann, E. Ribosomal synthesis of tricyclic depsipeptides in bloom-forming cyanobacteria. *Angew. Chem. Int. Ed.* **47**, 7756–7759 (2008).
- Knappe, T. A. *et al.* Isolation and structural characterization of capistruiin, a lasso peptide predicted from the genome sequence of *Burkholderia thailandensis* E264. *J. Am. Chem. Soc.* **130**, 11446–11454 (2008).
- Kalyon, B. *et al.* Plantazolicin A and B: Structure elucidation of ribosomally synthesized thiazole/oxazole peptides from *Bacillus amyloliquefaciens* FZB42. *Org. Lett.* **13**, 2996–2999 (2011).
- Scholz, R. *et al.* Plantazolicin, a novel microcin B17/streptolysin S-like natural product from *Bacillus amyloliquefaciens* FZB42. *J. Bacteriol.* **193**, 215–224 (2011).
- Arnison, P. G. *et al.* Ribosomally synthesized and post-translationally modified peptide natural products: Overview and recommendations for a universal nomenclature. *Nat. Prod. Rep.* **30**, 108–160 (2012).
- Budisa, N. *et al.* Proteins with beta-(thienopyrrolyl)alanines as alternative chromophores and pharmaceutically active amino acids. *Protein Sci. Publ. Protein Soc.* **10**, 1281–1292 (2001).
- Mendo, S., Faustino, N. A., Sarmiento, A. C., Amado, F. & Moir, A. J. G. Purification and characterization of a new peptide antibiotic produced by a thermotolerant *Bacillus licheniformis* strain. *Biotechnol. Lett.* **26**, 115–119 (2004).

39. Martin, N. I. *et al.* Structural characterization of lacticin 3147, a two-peptide lantibiotic with synergistic activity. *Biochemistry* **43**, 3049–3056 (2004).
40. Wiedemann, I. *et al.* The mode of action of the lantibiotic lacticin 3147 - a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. *Mol. Microbiol.* **61**, 285–296 (2006).
41. Oman, T. J. & van der Donk, W. A. Insights into the mode of action of the two-peptide lantibiotic haloduracin. *ACS Chem. Biol.* **4**, 865–874 (2009).
42. Zhao, X. & van der Donk, W. A. Structural characterization and bioactivity analysis of the two-component lantibiotic *Flv* system from a ruminant bacterium. *Cell Chem. Biol.* **23**, 246–256 (2016).
43. Wiedemann, I., Benz, R. & Sahl, H.-G. Lipid II-mediated pore formation by the peptide antibiotic nisin: a black lipid membrane study. *J. Bacteriol.* **186**, 3259–3261 (2004).
44. Morgan, S. M., O'Connor, P. M., Cotter, P. D., Ross, R. P. & Hill, C. Sequential actions of the two component peptides of the lantibiotic lacticin 3147 explain its antimicrobial activity at nanomolar concentrations. *Antimicrob. Agents Chemother.* **49**, 2606–2611 (2005).
45. Oman, T. J. *et al.* Haloduracin α binds the peptidoglycan precursor lipid II with 2:1 stoichiometry. *J. Am. Chem. Soc.* **133**, 17544–17547 (2011).
46. Caetano, T., Krawczyk, J. M., Mösker, E., Süßmuth, R. D. & Mendo, S. Heterologous expression, biosynthesis, and mutagenesis of type II lantibiotics from *Bacillus licheniformis* in *Escherichia coli*. *Chem. Biol.* **18**, 90–100 (2011).
47. Caetano, T., Krawczyk, J. M., Mösker, E., Süßmuth, R. D. & Mendo, S. Lichenicidin biosynthesis in *Escherichia coli*: *licFGEHI* immunity genes are not essential for lantibiotic production or self-protection. *Appl. Environ. Microbiol.* **77**, 5023–5026 (2011).
48. Basi-Chipalu, S. *et al.* Pseudomycoicidin, a class II lantibiotic from *Bacillus pseudomycoicoides*. *Appl. Environ. Microbiol.* **81**, 3419–3429 (2015).
49. Kuthning, A., Mösker, E. & Süßmuth, R. D. Engineering the heterologous expression of lanthipeptides in *Escherichia coli* by multigene assembly. *Appl. Microbiol. Biotechnol.* **99**, 6351–6361 (2015).
50. Schmidt, M. & de Lorenzo, V. Synthetic bugs on the loose: containment options for deeply engineered (micro)organisms. *Curr. Opin. Biotechnol.* **38**, 90–96 (2016).
51. Hoels, M. G. & Budisa, N. *In vivo* incorporation of multiple noncanonical amino acids into proteins. *Angew. Chem. Int. Ed.* **50**, 2896–2902 (2011).
52. Grasmann, H. *et al.* Inhalation of Moli1901 in patients with cystic fibrosis. *Chest* **131**, 1461–1466 (2007).
53. Boakes, S., Appleyard, A. N., Cortés, J. & Dawson, M. J. Organization of the biosynthetic genes encoding deoxyactagardine B (DAB), a new lantibiotic produced by *Actinoplanes liguriae* NCIMB41362. *J. Antibiot. (Tokyo)* **63**, 351–358 (2010).
54. Fox, J. L. Antimicrobial peptides stage a comeback. *Nat. Biotechnol.* **31**, 379–382 (2013).
55. Uhlig, T. *et al.* The emergence of peptides in the pharmaceutical business: From exploration to exploitation. *EuPA Open Proteomics* **4**, 58–69 (2014).
56. Field, D., Hill, C., Cotter, P. D. & Ross, R. P. The dawning of a 'Golden era' in lantibiotic bioengineering. *Mol. Microbiol.* **78**, 1077–1087 (2010).
57. Wals, K. & Ovaas, H. Unnatural amino acid incorporation in *E. coli*: Current and future applications in the design of therapeutic proteins. *Front. Chem.* **2**, 15 (2014).
58. McGann, P. *et al.* *Escherichia coli* harboring *mcr-1* and *bla_{CTX-M}* on a novel IncF plasmid: First report of *mcr-1* in the United States. *Antimicrob. Agents Chemother.* **60**, 4420–4421 (2016).
59. Zinner, S. H. The search for new antimicrobials: why we need new options. *Expert Rev. Anti Infect. Ther.* **3**, 907–913 (2005).
60. Khameneh, B., Diab, R., Ghazvini, K. & Fazly Bazzaz, B. S. Breakthroughs in bacterial resistance mechanisms and the potential ways to combat them. *Microb. Pathog.* **95**, 32–42 (2016).
61. Field, D., Cotter, P. D., Hill, C. & Ross, R. P. Bioengineering lantibiotics for therapeutic success. *Syst. Microbiol.* **5**, 1363 (2015).
62. Gust, B., Challis, G. L., Fowler, K., Kieser, T. & Chater, K. F. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc. Natl. Acad. Sci. USA* **100**, 1541–1546 (2003).

Acknowledgements

We sincerely thank Traudl Wenger for her support on the evolution of the expression strain used in this study. We acknowledge financial support from the Einstein Foundation supported ARTCODE consortium (A-2011-53), the 7th Framework program of the European Union funded METACODE consortium (FP7-KBBE-2011-5-CP-CSA) and the UniCat Cluster of Excellence at TU Berlin.

Author Contributions

A.K. designed and performed experiments, and analyzed all data. P.D. performed chemical synthesis of 4*H*-thieno[3,2-*b*]pyrrole. M.G.H. and S.O. generated the evolutionarily adapted strain and provided advice on handling and cultivation strategies. N.B. and R.D.S. contrived and supervised the project. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Kuthning, A. *et al.* Towards Biocontained Cell Factories: An Evolutionarily Adapted *Escherichia coli* Strain Produces a New-to-nature Bioactive Lantibiotic Containing Thienopyrrole-Alanine. *Sci. Rep.* **6**, 33447; doi: 10.1038/srep33447 (2016).



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2016