Supplementary Material

Ψ-footprinting approach for the identification of protein synthesis inhibitor producers

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**Supplementary Table S1:** Composition of the production media used for cultivation of the antibiotic producers. All quantities refer to 1 l.

| Medium  | Composition               | Amount          |
|---------|---------------------------|-----------------|
| NL500   | Glycerol                  | 10 g            |
|         | Starch, soluble           | 10 g            |
|         | Glucose                   | 10 g            |
|         | Fish Meal                 | 15 g            |
|         | Sea Salts                 | 10 g            |
|         | pH 8.0                    |                 |
| NL800   | Glucose                   | 5 g             |
|         | Glycerol                  | 10 g            |
|         | Starch, soluble           | 10 g            |
|         | Oatmeal, Holo             | 58 g            |
|         | Yeast extract             | 2 g             |
|         | NaCl                      | 1 g             |
|         | CaCO₃                     | 1 g             |
|         | pH 7.2                    |                 |
| MS (1)  | Mannitol                  | 20 g            |
|         | Oatmeal, Holo             | 20 g            |
| R5 (1)  | Sucrose                   | 103 g           |
|         | Glucose                   | 10 g            |
|         | K₂SO₄                     | 0.25 g          |
|         | MgCl₂                     | 10.12 g         |
|         | Casamino acids            | 0.1 g           |
|         | Yeast extract             | 5 g             |
|         | TES                       | 5.73 g          |
|         | CaCl₂ x 2H₂O              | 2.94 g          |
|         | KH₂PO₄                    | 0.05 g          |
|         | L-proline                 | 3 g             |
|         | Trace element solution    | 2 ml            |
|         | pH 7.4                    |                 |
| Trace element solution | Fe x 6H₂O                  | 200 mg          |
|         | Na₂B₄O₇ x 10H₂O           | 10 mg           |
|         | (NH₄)₆Mo₇O₂₄ x 4H₂O       | 10 mg           |
|         | CuCl₂ x 2H₂O              | 10 mg           |
|         | MnCl₂ x 4H₂O              | 10 mg           |
|         | ZnCl₃                     | 40 mg           |
**Supplementary Table S2:** Genome sequence data for all analyzed strains of the Tübingen strain collection.

| Sequencing method        | Tü 2108 | Tü 2975 | Tü 3180 | Tü 6430 | A 4/2 | KNN 49.3e |
|--------------------------|---------|---------|---------|---------|-------|-----------|
|                          | Illumina| PacBio  | PacBio  | PacBio  | Illumina| PacBio    |
|                          | MiSeq   | RSII    | RSII    | RSII    | MiSeq  | RSII      |
| **De novo Assembly**     |         |         |         |         |       |           |
| contig(s)                | 189     | 1       | 2       | 10      | 144    | 1         |
| draft sequence (in bp)   | 7,920,474 | 7,623,788 | 8,634,962 | 8,176,333 | 8,295,973 | 7,285,716 |
| N50 contig               | 219,353 | 7,623,788 | 8,390,116 | 5,909,951 | 235,910 | 7,285,716 |
| maximal length           | 657,624 | 7,623,788 | 8,390,116 | 5,909,951 | 511,072 | 7,285,716 |
| average length           | 64,286  | 7,623,788 | 4,317,481 | 817,633  | 91,077  | 7,285,716 |
| coverage depth           | 129     | 87      | 95      | 50      | 105    | 123       |
| average. G+C content (in %) | 70.81  | 71.04   | 72.97   | 72.63   | 70.27   | 71.85     |
| **Genome Annotation**    |         |         |         |         |       |           |
| coding sequences (CDSs)  | 7,064   | 6,950   | 7,470   | 8,019   | 7,398   | 7,005     |
| tRNA                     | 82      | 80      | 97      | 94      | 78      | 70        |
| rRNA                     | 9       | 18      | 18      | 18      | 9       | 9         |
### Supplementary Figure S1: List of predicted secondary metabolite gene clusters for strain Tü 2108 identified by antiSMASH 6.0 analysis.

| Region | Type               | From    | To       | Most similar known cluster                  | Similarity |
|--------|--------------------|---------|----------|----------------------------------------------|------------|
| 4.1    | siderophore Δf'    | 132,675 | 144,459  | desferrioxamine B Δf'                        | 80%        |
| 4.2    | lanthipeptide Δf'  | 207,057 | 230,198  | niphymycin C-E Δf'                          | 6%         |
| 6.1    | bacteriocin Δf'    | 44,627  | 54,830   | Polypeptide                                   |            |
| 6.2    | lanthipeptide Δf'  | 115,833 | 141,053  | Polypeptide                                   |            |
| 6.3    | thiopenone Δf', LAP Δf' | 406,810 | 497,811 | berininmycin A Δf'                           | 100%       |
| 10.1   | butyrolactone Δf'  | 90,791  | 101,717  | lactonamycin Δf'                             | 3%         |
| 18.1   | terpene Δf'        | 144,069 | 165,142  | Polypeptide                                   |            |
| 19.1   | terpene Δf'        | 87,908  | 108,900  | Polypeptide                                   |            |
| 19.2   | NRPS Δf'           | 396,004 | 437,854  | Polyketide:Type II + Saccharide:Hybrid/tailoring | 16%        |
| 22.1   | butyrolactone Δf'  | 10,280  | 21,194   | NRP                                          | 9%         |
| 23.1   | lanthipeptide Δf'  | 1       | 21,233   | NRP                                          | 14%        |
| 24.1   | T3PKS Δf'          | 81,371  | 102,495  | Tetronas Δf'                                 | 3%         |
| 24.2   | melanin Δf'        | 175,968 | 188,069  | Other                                        | 11%        |
| 25.1   | T2PKS Δf', terpene Δf' | 101,514 | 136,384 | SPORE PIGMENT Δf'                           | 100%       |
| 25.2   | NRPS Δf'           | 12,002  | 62,903   | Other                                        | 83%        |
| 29.1   | NRPS Δf'           | 1,773   | 59,016   | Other                                        | 1%         |
| 34.1   | NRPS Δf'           | 55,138  | 90,630   | Other                                        | 64%        |
| 35.1   | terpene Δf'        | 1,946   | 13,238   | Other                                        | 100%       |
| 35.2   | NRPS Δf'           | 224,143 | 253,561  | NRP: Lipopeptide + Polyketide:Modular type I + Saccharide:Hybrid/tailoring | 9%         |
| 39.1   | NRPS Δf'           | 110,514 | 136,384  | NRP                                          | 10%        |
| 39.2   | melanin Δf'        | 243,028 | 274,370  | NRP                                          | 3%         |
| 39.3   | oligosaccharide Δf' | 149,593 | 159,970  | Other                                        | 61%        |
| 41.1   | terpene Δf'        | 50,700  | 77,357   | Other                                        | 40%        |
| 41.2   | NRPS Δf'           | 156,102 | 210,128  | Other                                        | 1%         |
| 45.1   | PKS-like Δf'       | 62,303  | 103,322  | Other                                        | 100%       |
| 45.2   | NRPS Δf'           | 224,143 | 253,561  | Other                                        | 9%         |
| 48.1   | T1PKS Δf', terpene Δf' | 104,253 | 25,382   | Other                                        | 61%        |
| 48.2   | bacteriocin Δf'    | 104,323 | 114,550  | Other                                        | 11%        |
| 48.3   | hgl-EKS Δf', T1PKS Δf' | 122,159 | 126,855  | Other                                        | 19%        |
| 50.1   | siderophore Δf'    | 1       | 1,012    | Other                                        | 50%        |
| 53.1   | T1PKS Δf'          | 1       | 57,245   | Other                                        | 40%        |
| 64.1   | terpene Δf'        | 91,851  | 106,441  | Other                                        | 10%        |
| 67.1   | NRPS Δf'           | 7,567   | 28,689   | Other                                        | 15%        |
| 68.1   | T1PKS Δf'          | 1       | 13,178   | Other                                        | 44%        |
| 71.1   | NRPS Δf'           | 1       | 14,779   | Other                                        | 9%         |
| 73.1   | T1PKS Δf'          | 1       | 10,243   | Other                                        | 10%        |
Supplementary Figure S2: (A) HPLC spectrum of the R5 culture extract from strain Tü 2108. Wavelength monitoring was performed at 210 nm. Berninamycin C specific peak at retention time (RT) 9.7 min. (B) MS data referring to peak at RT 9.7 min. Berninamycin C peaks (1077.4 [M+H]+, m/z 1099.3 [M+Na]+; (2)) at RT 11.1 min are shown. (C) MS reference data from berninamycin C (1077.4 [M+H]+, m/z 1099.4 [M+Na]+) at RT 10.9 min and corresponding chemical structure from the Dictionary of Natural Products (3).
Berninamycin A: $R_1 = \text{Dha}_2-\text{Dha}_1-\text{NH}_2$, $R_2 = \text{OH}$
Berninamycin B: $R_1 = \text{Dha}_2-\text{Dha}_1-\text{NH}_2$, $R_2 = \text{H}$
Berninamycin C: $R_1 = \text{Dha}_2-\text{NH}_2$, $R_2 = \text{OH}$
Berninamycin D: $R_1 = \text{NH}_2$, $R_2 = \text{OH}$
Berninamycin E: $R_1 = \text{Dha}_2-\text{ala}-\text{NH}_2$, $R_2 = \text{OH}$

**Supplementary Figure S3:** Chemical structure of berninamycin derivatives. Berninamycin A - hydroxy group at Val7; berninamycin B - missing hydroxy group at Val7; berninamycin C - one missing dehydroalanine residue at the N-terminus, berninamycin D - two missing dehydroalanine residues at the N-terminus; berninamycin E - in place of the N-terminal dehydroalanine is an alanine residue (2, 4) Purple ellipses indicate the position of the altered berninamycin residues. Dehydroalanine (Dha), alanine (Ala), valine (Val).

**Supplementary Figure S3: Additional information:** Berninamycin BGC comparisons provide information on the production of compound derivatives:

Tü 2108 was found to produce berninamycin C exclusively (5) (Figure S2). *S. bernensis* produces berninamycin A-D, with berninamycin A as the major compound (6), whereas heterologous expression of the *berA*-J gene cluster from *S. bernensis* in *Streptomyces lividans* resulted in a stable berninamycin A production, while berninamycin B and D were only produced in tiny amounts and berninamycin C was not produced at all (6). *S. atroolivaceus* produces berninamycin A and E, with berninamycin A as the major compound (4, 7). Differences in berninamycin derivative production might be related to the *berA* core-prepeptide coding sequence. Malcolmson *et al.*, 2013 showed that mutations in the BerA core-prepeptide sequence result in the production of berninamycin analogs, e.g. the introduction of a T3A mutation in the *berA* gene sequence resulted in...
the production of different compounds in *S. lividans*, including a substance with a C-terminal amide, an unmodified Val7 residue, and an N-terminal alanine residue (6). Thus, *berA* mutations cause structural changes in the BerA protein, which are responsible for the formation of the various different berninamycin derivatives (2, 4) (Figure S3). Since the similarity between BerA from *S. bernensis* and ORF 01237 from Tü 2108 is rather low (only 65%), the exclusive berninamycin C production of Tü 2108 is likely to be the result of the specific core prepeptide sequence encoded by ORF 01237. Thus, the results presented in this study show that Tü 2108 is a new producer strain of berninamycin and provide an explanation for the high berninamycin C production rate of Tü 2108.

**Supplementary Table S3**: Sum of the 790 detected hit genes from 35 analyzed genome sequences of known PSI producers and their assignment to the following criteria for self-resistance genes: phylogenetic incongruence of core gene, gene duplication, gene localization in a BGC. Shown are the gene numbers and the percentage for each criterium. Some of the genes fulfill several criteria; therefore, the total numbers and percentage of the genes exceeds 790 and 100%, respectively.

|                  | No. of genes | Percentages of genes |
|------------------|--------------|----------------------|
| Phylogeny        | 612          | 77 %                 |
| Duplication      | 338          | 43 %                 |
| BGC              | 205          | 26 %                 |

**Supplementary Table S4**: Occurrence of ‘resistance indicator’ (RI) genes within the 406 analyzed genome-sequenced strains from the DSMZ strain collection using the Ψ-footprinting method. Numbers and the percentages of all RI genes are shown.

| No. of RI gene(s) | No. of strains | Percentages of strains |
|-------------------|----------------|------------------------|
| 0                 | 265            | 65%                    |
| 1                 | 36             | 9%                     |
| 2                 | 55             | 14%                    |
| 3                 | 22             | 6%                     |
| 4                 | 18             | 4%                     |
| 5                 | 9              | 2%                     |
| 6                 | 0              | 0%                     |
| 7                 | 1              | 0.2%                   |
Supplementary Figure S4: *In vitro* transcription/translation assay performed with culture extracts in MS (A) and R5 (B) media from 4, 7, and 10 days. PSI antibiotics, tet₁₅ and apra₅₀ = positive control (orange), medium extracts, extracts of M1146 = negative control (green), and extracts of DSM cultures (blue). Results are shown for samples, which did not lead to an inhibition of the ivTT assay. Measurements have been performed in triplicate using the same preparation of S12 extract.

Supplementary Figure S5: *In vitro* transcription/translation assay performed with culture extracts in NL800 (A), MS (B), and R5 (C) media from days 4, 7, and 10. Only strains are shown, which were able to grow in the respective media. PSI antibiotics, tet₁₅ and apra₅₀ = positive control (orange), medium extracts, extracts of M1146 = negative control (green), and extracts of DSM cultures (blue). Measurements have been performed in triplicate using the same preparation of S12 extract.
Supplementary Figure S5 - Additional information: Description of additional *in vitro* transcription/translation (ivTT) assay results:

*ivTT* assays with extract samples of strain DSM 44944 incubated for 10 days in NL800 medium resulted in inhibition of GFP expression with values of 29% maximal fluorescence (Figure 6, Supplementary Figure S5). Extract samples of strain DSM 45888, DSM 45258, and DSM 43821 grown for 7 days in NL800 showed similar values (28%, 26%, and 24%, respectively) (Figure 6, Supplementary Figure S5). Inhibition of the *ivTT* assay suggests that DSM 44944, DSM 45888, DSM 45258, and DSM 43821 produce a PSI when grown in NL800, whereby the time point for best production varies.

*ivTT* assays with culture extracts of DMS 44073 and DMS 45079 grown for 7 days in MS medium resulted in the inhibition of GFP expression with fluorescence values of 35% and 31%, respectively, indicating the presence of a PSI in both extracts (Figure 6, Supplementary Figure S5). Culture extracts of DMS 45657 grown for 7 days in R5 medium yielded similar inhibition results with maximal fluorescence values of 32%.

*ivTT* assays with culture extracts of DSM 43813, DSM 45079, DSM 45408, DSM 25218, and DSM 44771 grown in R5 resulted in the lowest fluorescence values (<20% maximal fluorescence), which indicates strong inhibition of GFP expression (Figure 6, Supplementary Figure S5). Extracts from strains DSM 43813 and DSM 45079 grown for 7 and 10 days showed an inhibiting effect on GFP expression with fluorescence values of 17% and 12%, respectively.

Supplementary Figure S6: *In vitro* transcription/translation assay performed with extract samples from strains of the Tübingen strain collection, which have not been prioritized with the Ψ-footprinting method. Apra50 = positive control. Extracts have been generated from strains of the Tübingen strain collection cultivated in R5 medium for 7 days. Measurements have been performed in triplicate using the same preparation of S12 extract.
**Supplementary Figure S7**: *In vitro* transcription/translation assay performed with R5 culture extract of DSM 45079 from day 10. Displayed are the fractions generated by semi-preparative HPLC (blue). PSI antibiotic tet₁₅ = positive control (orange). Fraction having the greatest inhibition is shown in red. Measurements have been performed in triplicate using the same preparation of S12 extract.

**Supplementary Figure S8**: *In vitro* transcription/translation assay performed with R5 culture extract of DSM 45408 from day 10. Displayed are the fractions generated by semi-preparative HPLC (blue). PSI antibiotic apra₅₀ = positive control (orange). Fraction having the greatest inhibition is shown in red Measurements have been performed in triplicate using the same preparation of S12 extract.
Supplementary Figure S9: *In vitro* transcription/translation assay performed with R5 culture extract of DSM 25218 from day 7. Displayed are the fractions generated by semi-preparative HPLC (blue). PSI antibiotic apra<sub>50</sub> = positive control (orange). Fraction having the greatest inhibition is shown in red. Measurements have been performed in triplicate using the same preparation of S12 extract.

Supplementary Figure S10: *In vitro* transcription/translation assay performed with R5 culture extract of DSM 44771 from day 4. Displayed are the fractions generated by semi-preparative HPLC (blue). PSI antibiotic apra<sub>50</sub> = positive control (orange). Fraction having the greatest inhibition is shown in red. Measurements have been performed in triplicate using the same preparation of S12 extract.
HRMS analysis

High resolution mass spectrometry was done with a Bruker Elute UHPLC 1300 coupled with a QTOF (Impact II, Bruker). 2 µl extract were injected on a Kinetex 100 C18 column (50 x 2.1 mm, 1.7 µm, Phenomenex) and eluted by a linear gradient from 90 % solvent A (water with 0.1 % formic acid) to 100 % solvent B (acetonitrile with 0.1 % formic acid) over 10 minutes with a flow rate of 0.5 ml/min. QTOF parameters were as follows: capillary voltage 4500 V, nebulizer 2 bar, drying gas 8 l/min, dry heater 220 °C. Data evaluation was conducted with Bruker Compass DataAnalysis 5.2.

Amicoumacin A

Supplementary Figure S11: HRMS data of sample F8 from DSM 45408. Positive extracted ion chromatograms (EICs) and MS1 of amicoumacin A (above) and B (below). Expected mass for amicoumacin A (C20H30N3O7) is 424.2078 and for amicoumacin B (C20H29N2O8) 425.1918, error rates are -0.1 ppm.
Supplementary Figure S12: Whole-genome sequence-based phylogenetic tree generated with the TYGS web server for the amicoumacin B producer strains DSM 44771, DSM 43813, DSM 25218, DSM 45408, and closely related type strains. The known amicoumacin producers *Bacillus pumulus* and *Nocardia jinanensis* were added manually to the reference strains. All amicoumacin producer strains are marked in bold. Tree inferred with FastME from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula $d_5$. The numbers above branches are GBDP pseudo-bootstrap support values >62% from 100 replications, with an average branch support of 85.3%.
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