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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of all covariates tested
☒	☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Plate-based fluorescence measurements were collected using Softmax Pro 7.0.3 software with a Spectramax i3x plate reader. Optical density measurements were collected using either the Softmax Pro 7.0.3 software with a Spectramax i3x plate reader or Genesys software (Version 2.6) with a BioMate 160 UV-Vis spectrophotometer. HPLC data were collected using an Agilent 1100 Infinity model equipped with a Zorbax Eclipse Plus-C18 column (part number: 959701-902, 5 µm, 95Å, 2.1 x 150 mm) and Agilent ChemStation (Version C.01.10) software. LC-MS/MS data was collected using an Orbitrap Eclipse MS (Thermo Scientific) with an Ultimate 3000 nano-LC and a FAIMS Pro Interface (Thermo Fisher Scientific). UPLC-MS data were collected using Waters AQUITY Arc UPLC H-Class with a diode array coupled to a Waters AQUITY QDa Mass Detector or an Agilent 1290 UHPLC-MS equipped with a 1260 DAD and 6125 SQD. Intact protein MS was performed in ESI positive mode using a Waters Acquity UPLC protein BEH C4 columns 1.7um 2.1m X 50 mm.
Data analysis	LC data were analyzed using Agilent ChemStation (Version C.01.10) software. Microsoft Excel was used to analyze plate reader data, and Graphpad Prism was used to generate plots and calculate standard deviations. Whole protein and small molecule LC/MS data was analyzed using MassLynx (Version 4.2) software. Tryptic digestion LC-MS/MS data was analyzed using MaxQuant-Andromeda software (v2.5.1.0).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The datasets generated during and/or analyzed during the current study are contained in the published article (and its Supplementary Information) or are available from the corresponding author on reasonable request.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	N/A
Reporting on race, ethnicity, or other socially relevant groupings	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample sizes were selected based on acceptable convention in the field where 3 replicates is standard practice for plate reader experiments (examples include: (i) doi:10.1038/s41467-021-25691-4 and (ii) doi:10.1038/nbt.3372) and HPLC analysis of metabolites produced via fermentation (examples include: (i) doi:10.1038/ncomms11709 and (ii) doi: 10.1038/s41589-020-00684-4). Single purified protein samples were used for intact LC/MS and for tryptic digestion LC-MS/MS. Previous studies investigating nsAA incorporation only report single replicates for quantification/confirmation (examples include: (i) doi:10.1002/cbic.201600448 (ii) doi: 10.1039/c7ob00582b (iii) doi:10.1021/acschembio.9b01026). No calculation was used to determine sample size, but experiments were repeated to increase replicability.
Data exclusions	No data were excluded.
Replication	All attempts to replicate were successful, and the number of replicates is reported for each experiment. Some full experiments were performed in replicates on separate days, exhibiting similar results with a single day's set of replicates chosen here.
Randomization	Experimental samples using the same strain but coming from different groups were generated from the same source. If one strain was exposed to +nsAA and -nsAA conditions, a single culture of that strain was split immediately before nsAA was added. For this study, randomization was not relevant, as all bacterial cells were analyzed equally.
Blinding	All experiments were unblinded as there would be difficulty fully blinding studies to ensure proper growth and media conditions, but is unlikely researcher bias in would result in replicated effects.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used: Horseradish peroxidase-conjugated 6*His, His-tag monoclonal antibody was used from Proteintech (catalog number: HRP-66005; clone number: 21006227)

Validation: Validation was performed by vendor: "Recombinant protein were subjected to SDS PAGE followed by western blot with HRP-66005 (6*His, His-Tag antibody) at dilution of 1:10000 incubated at room temperature for 1.5 hours."

Plants

Seed stocks: N/A

Novel plant genotypes: N/A

Authentication: N/A