Supplementary Information

Host-dependent resistance of Group A *Streptococcus* to sulfamethoxazole mediated by a horizontally-acquired reduced folate transporter.

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Supplementary Note 1

Metabolic profiling of MH-Bm and MH-Ox media. The differences in the susceptibility of TB08-2-14 to SXT on MHF-Ox and MHF-Bm media suggest that there are differences in the composition of MH media from these different suppliers. To investigate this, we first examined the composition of MH base medium from each supplier using nuclear magnetic resonance (NMR) spectroscopy. Using this approach, we found several differences in the composition of MH base medium from each supplier, with the MH-Ox medium containing higher levels of adenine, uracil and TRIS, while the MH-Bm medium contained higher levels of uridine, glucose and an unknown guanine moiety containing compound (Supplementary Fig. 11). However, we were unable to identify any major differences in folate pathway compounds using this approach. To further investigate the composition of MH-Ox and MH-Bm, we performed untargeted metabolomics analysis using reversed-phase LC-MS/MS. Confirming the results obtained with NMR spectroscopy (above), we observed multiple differences in the composition of each medium following positive and negative ionisation (Supplementary Fig. 12, Supplementary Table 10), but did not detect differences in folate pathway intermediates. Untargeted LC-MS profiling followed by multivariate statistical analysis (principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) and database peak annotation revealed significant differences in a number of analytes (Supplementary Fig. 12), including higher levels of guanosine, uridine, methionine and peptides Pro-Ile-Ile and Pro-Val-Ile in the MH-Bm medium, with the MH-Ox medium containing higher levels of adenine, uracil, pyrrolidonecarboxylic acid and citric acid (Supplementary Table 10). Further comparison of spectra against reference standards for folate pathway intermediates revealed no significant differences other than a slight increase in 5,10-methylene-THF in MH-Bm (Supplementary Fig. 13). Thus, while there were major differences in the gross composition of MH media from each supplier, we were unable to identify substantial differences in the concentration of folic acid or reduced folate derivatives that might explain the relative performance of each media for detecting ThfT-mediated SMX resistance.

Supplementary Methods

1H NMR sample preparation: The cell media were thawed at 6 °C for 2 h. 560 µL of the cell medium was directly injected into the NMR tube. Afterwards 40 µL of D₂O were added and the mixture was mixed thoroughly. All samples were prepared in standard 5 mm outer diameter NMR tubes.

1H NMR spectroscopy data acquisition and processing parameters: NMR spectroscopic analyses were performed on a 600 MHz Bruker Avance III HD spectrometer, equipped with a 5 mm BBI probe and fitted with a Bruker SampleCase set to 6 °C. All spectra were acquired at 300 K and the correct temperature was ensured with a 99.8% Methanol-d₄ sample prior to measurement. The standard one-dimensional (1D) experiments with solvent suppression (pp: noesygppr1d) were acquired with 128 scans (+4 dummy scans), 64k data points, relaxation delay of 4.0 s, and a spectral width of 20 ppm resulting in a total experiment time of 14 min 53s. The spiking experiments were acquired with under the same conditions after addition of small quantities of the compound in question. Time domain data were Fourier transformed and processed manually using Bruker Topspin™ 3.6.2 or Bruker Topspin™ 4.1.3 to obtain phase and baseline corrected spectra. An exponential line broadening of 0.3 Hz was applied to the free induction decays (FID).
Standard preparation. Reference standards for each standard was from suppliers as indicated in Supplementary Table 6. Stock solutions were prepared at 1 mg/mL in DMSO, and further diluted to 10 µg/mL in methanol containing 0.1 % butylated hydroxytolunene (BHT) for LC-MS analysis.

Sample preparation. Media samples were filtered using a 3 kDa MWCO filter (Millipore Centriprep) and either directly injected for the detection of targeted tetrahydrofolic acid metabolites, or diluted 1 in 10 (v/v) with LC-MS grade water for untargeted profiling. Preparation was identical for both methods.

LC-MS method. Chromatographic separation was performed on a Waters Acquity I-class UPLC system (Waters, Wilmslow, UK), using a Waters Cortecs T3, 100mm x 2.1mm x 1.6 µm (Waters, Wilmslow, UK) kept at 40 ºC. Mobile phase A consisted of water containing 0.1 % formic acid, and mobile phase B was acetonitrile containing 0.1 % formic acid. The flow rate was set at 0.25 mL/min. Gradient elution was performed with initial conditions starting at 0 % B, held for 1.25 minutes, increasing to 10 % B at 6.00 minutes, 25 % B at 8.00 minutes and 75 % B at 10.50 minutes, followed by a wash step to 95 % B until 11.50 minutes. At 11.50 minutes, the flow was returned to initial conditions (0% B), allowing for 3.50 minutes of re-equilibration time. A 10 µL injection volume was performed. Mass spectrometry was performed on a Bruker Impact II QTOF-MS (Bruker, Bremen, Germany) with electrospray ionisation operated in positive and negative mode. The capillary voltage was set at 4500V in positive ionisation mode and 3500 V in negative ionisation mode. Drying gas was set at 10 L/min, gas temperature was 220 ºC, nebuliser pressure was 2.2 bar and the end plate offset was 500 V. MS1 scan rate was set at 12 Hz. Auto MS/MS was enabled, with 4 precursors automatically selected and data collected at a scan rate of 25 Hz per scan cycle, resulting in a total scan cycle time (MS1 + auto MS/MS) of 0.2 seconds. An internal calibration was performed by injection of 5 mM sodium formate solution in water:isopropanol (50:50 v/v) at the beginning of every run. Mass spectrometric data were collected with Compass HyStar 5.1 and O-TOF Control version 5.2. Data were reviewed using Compass DataAnalysis 5.2 (Bruker Daltonics, Bremen, Germany) and pre-processed in Metaboscape 2022 B. Multivariate statistical analysis was performed using SIMCA® 17.0 (Sartorius AG, Göttingen, Germany).
**Amino acid sequence alignment of FolP and associated SMX MICs for GAS strains.** Alignments were performed with CLUSA TLW using translated *folP* gene sequences. Residues identified by structural\textsuperscript{16} and biochemical\textsuperscript{17} studies as being important for SMX resistance are highlighted in red. Genbank accessions were as follows: M6\textsubscript{JRS4} (AK176294.1), M1\textsubscript{5448} (AKK70503.1). SMX MIC values are from Supplementary Table 1. Shading indicates level of sensitivity/resistance: Green, sensitive (SMX MIC <100 µg/ml); Light red, low resistance (SMX MIC 100-500 µg/ml); Dark red, highly resistant (SMX MIC >500 µg/ml).
Supplementary Fig. 2.

**dfrF confers very high TMP-resistance.** (a) Comparison of TMP MICs for *dfrF*-positive and *dfrG*-positive GAS strains measured on gradient agar plates. The TMP gradient range is indicated below each image. *dfrF*-positive GAS strains exhibit higher TMP resistance than *dfrG*-positive GAS strains. (b) Schematic showing hypothetical effect of DfrF and DfrG on synergy of SMX and TMP. Limits of detection of SMX (1024 µg/ml) and TMP (32 µg/ml) Etest assays are indicated with red dashed lines. Relative TMP resistance provided by DfrG and DfrF are indicated with light blue dashed lines. The red zone below the synergy curve represents SXT inhibition of growth, while the white zone above the curve represents bacterial growth.
Supplementary Fig. 3.

*In vitro* evolution of GAS strain TB08 on SXT gradient plates. Growth of TB08 on SXT gradient plates following successive rounds of selection on SXT gradient plates. Each gradient plate demonstrates the bacterial growth following every second round of selection.
Supplementary Fig. 4.

SXT Etest results of TB08 and TB08-2-14 (round 14, Exp 2) strains on different formulations of agar. Results are mean ± SD of three biological replicates. HB, 5% horse blood; LHB, 2.5% lysed horse blood. Differences assessed using a two-tailed, unpaired Student's t-test for MHF-Bm comparison. For all other comparisons, a one-sample t-test was used to determine differences from the maximum resolution of the Etest assay (32 µg/ml).
Supplementary Fig. 5.

Alignment of ThfT with FolT S component proteins from *Lacticaseibacillus casei* (*Lcas*), *Lacticaseibacillus casei* (*Lmes*), and *Leuconostoc mesenteroides* (*Lmes*). Alignment was performed using ClustalW, with identical residues shaded black and similar residues shaded grey. Lcas, *Lacticaseibacillus casei*; Lmes, *Leuconostoc mesenteroides*. 
**Supplementary Fig. 6.**

**ThfT confers SXT resistance in the presence of exogenous THF.** Dose-response curves for the impact of exogenous THF on SXT resistance of M$_6^{JRS4}$, TB08 and TB08-2-14 determined by broth microdilution. MICs are expressed as a percentage the maximum SXT concentration examined in this assay (50 µg/ml). Data from three biological replicates are presented as mean values ± SEM. EC$_{50}$ values were calculated using Graphpad Prism software.
Supplementary Fig. 7.

**ThfT does not confer SXT resistance in the presence of exogenous folic acid.** Susceptibility of GAS strains in the presence of exogenous folic acid as determined with Etest strips on MHF-Bm agar. Results are representative of two independent experiments.
Supplementary Fig. 8.

**GAS proteins mapped onto the one carbon Kegg pathway map.** Green boxes represent enzymes that have homologues in the GAS strain MGAS5005 genome. Image was downloaded from the KEGG database\(^4\) ([https://www.genome.jp/kegg-bin/show_pathway?spz00670](https://www.genome.jp/kegg-bin/show_pathway?spz00670)) on 24 August 2022. Identical results are obtained with other GAS strains.
Supplementary Fig. 9.

Schematic showing hypothetical effect of reduced folate compounds (e.g. THF) on synergy of SMX and TMP for GAS strains containing either \textit{dfrG} (blue circles) or \textit{dfrF} (magenta circles). Limits of detection of SMX (1024 µg/ml) and TMP (32 µg/ml) Etest assays are indicated with red dashed lines. The zone below the dashed curve represents SXT inhibition of growth, while zone above the curve represents bacterial growth.
Overview of canonical antibiotic resistance mechanisms and comparison with SMX resistance mediated by ThfT. Red, antibiotic; Green, antibiotic target; Blue, AMR protein. Adapted from references 3-4. Created with BioRender.com. AST, antibiotic susceptibility testing.
Supplementary Fig. 11.

NMR spectroscopic measurements of MH-Ox and MH-Bm media. (a) 1D $^1$H with solvent pre-saturation for the MH-Bm medium. The aromatic region (left) is scaled up by a factor of ten compared to the aliphatic region (right) of the spectrum. (b) 1D $^1$H with solvent pre-saturation for the MH-Ox medium. The aromatic region (left) is scaled up by a factor of ten compared to the aliphatic region (right) of the spectrum. (c) Direct comparison of the two growth media MH-Bm (black) and MH-Ox (red) focussing on the proton NMR regions that showed major differences in peaks and/or peak intensities. Corresponding structures of the molecules or moieties are given next to the peak(s) in question and the proton which yields the peak is presented in bold. The structures (from left to right) are: Adenine, Guanine moiety containing compound (unknown), Uridine, Uracil, Glucose and TRIS.
Supplementary Fig. 12.

Untargeted LC-MS analysis of MH-Ox and MH-Bm media. (a) Comparison of the reversed-phase LC-MS base peak chromatograms in positive ionisation mode (MH-Bm orange, MH-Ox, blue). (b) Comparison of the reversed-phase LC-MS base peak chromatograms in negative ionisation mode (MH-Bm orange, MH-Ox, blue). (c) Principal component analysis (PCA) scores plot showing distinct separation resulting from replicate analysis of MH-Bm (orange) and MH-Ox (blue) media in LC-MS positive ionization mode. (d) Orthogonal projections to latent structures discriminant analysis (OPLS-DA) scores plot generated with data from LC-MS positive ionisation analysis of MH-Bm (orange) and MH-Ox (blue) media. (e) OPLS-DA S-plot generated from OPLS-DA of LC-MS positive ionisation data with the top 10 most discriminating features highlighted in red (Supplementary Table 10). (f) Principal component analysis (PCA) scores plot showing distinct separation resulting from replicate analysis of MH-Bm (orange) and MH-Ox (blue) media in LC-MS negative ionization mode. (g) Orthogonal projections to latent structures discriminant analysis (OPLS-DA) scores plot generated with data from LC-MS negative ionisation analysis of MH-Bm (orange) and MH-Ox (blue) media. (h) OPLS-DA S-plot generated from OPLS-DA of LC-MS negative ionisation data with the top 10 most discriminating features highlighted in red (Supplementary Table 10).
Targeted LC-MS analysis of folate pathway intermediates in MH-Ox and MH-Bm media.

Extracted ion chromatograms (EIC) generated from reversed-phase LC-quadrupole-time-of-flight (QToF)-MS positive ionisation analysis of folate pathway intermediate reference standards at 10 µg/mL, MH-Bm and MH-Ox media.
**Supplementary Table 1.**

| Strain | Lab ID | emmST | MLST | Location | SMX  | TMP  | SXT  | dfrG | dfrF | dyr | I100L | thfT |
|--------|--------|-------|------|----------|------|------|------|------|------|-----|-------|------|
| M1\(^{5448}\) | 5448   |   | 28   | USA      | 1.5  | 0.19 | 0.016 |
| M6\(^{JRS4}\) | JRS4   |   | 37   | USA      | 6    | 0.125| 0.016 |
| TB01   | 838    | 49.4 | 534  | India    | >1024| >32  | >32  |
| TB02   | 894    | 102.2| 489  | India    | 48   | 6    | 0.38 |
| TB07   | 981    | 102.2| 349  | India    | 64   | 4    | 0.25 |
| TB08   | 1001   | 4.2  | 289  | India    | >1024| >32  | 0.19 |
| TB09   | 1002   | 49   | 371  | India    | >1024| >32  | 4    |
| TB10   | 1017   | 44   | 351  | India    | >1024| >32  | 1.5  |
| TB11   | 1020   | 11   | 293  | India    | 32   | >32  | 0.5  |
| TB12   | 1024   | 22.8 | 360  | India    | 0.5  | >32  | 0.004|
| TB13   | 1033   | 102.2| 349  | India    | 64   | 1.5  | 0.38 |
| TB15   | 1380   | 113  | 677  | India    | 48   | 1    | 0.125|
| TB16   | 6653   | 28   | 458  | Germany  | 1    | >32  | 0.047|
| TB17   | 6666   | 81   | 290  | Germany  | 96   | >32  | 0.5  |
| TB18   | 6679   | 81.2 | 290  | Germany  | 64   | >32  | 0.38 |
| TB19   | 7794   | st854| -**  | Germany  | >1024| >32  | >32  |
| TB21   | 8970   | 3    | 406  | Germany  | 2    | 0.5  | 0.012|
| TB23   | 9116   | 113  | 677  | Germany  | 48   | 2    | 0.19 |
| TB24   | 9118   | 76   | 378  | Germany  | >1024| 2    | 0.047|
| TB27   | 9386   | 3    | 15   | Germany  | 6    | 2    | 0.032|
| TB28   | 9395   | 3    | 15   | Germany  | 6    | 2    | 0.047|
| TB31   | 10009  | 49   | 840  | Germany  | 128  | >32  | 2    |
| TB33   | 10964  | 27   | 1163 | Germany  | >1024| 1    | 0.032|
| TB34   | 10967  | 28   | 371  | Germany  | 128  | >32  | 2    |

Susceptibility of GAS strains to TMP, SMX and SXT. Control strains M1\(^{5448}\) and M6\(^{JRS4}\) are included for comparison. MICs to each antibiotic was determined by determined with Etest strips on MHF-Bm agar. Each MIC value is the median value from three biological replicates. Source data are provided as a Source Data file. Shading indicates level of sensitivity/resistance: Green, sensitive (SMX MIC <100 µg/ml; TMP/SXT MIC <2 µg/ml); Light red, low resistance (SMX MIC 100-500 µg/ml; TMP/SXT MIC 2.0-10 µg/ml); Dark red, highly resistant (SMX MIC >500 µg/ml; TMP/SXT MIC >10 µg/ml). Presence (black) and absence (white) of TMP resistance genes in genome sequences is indicated to the right, as determined by BLASTN. emmST, emm gene sequence type. MLST, multi-locus sequence type. * Isolates from reference 13 (TB01-TB15) and reference 15 (TB16-TB34). ** TB19 does not belong to any currently-annotated MLST.
Effect of exogenous THF on the susceptibility of GAS strains to SXT. Control strain M$^6_{JRS4}$ is included for comparison. MICs to each antibiotic was determined by determined with Etest strips on MHF-Bm agar. Each MIC value is the mean value ± SD from three biological replicates. Source data are provided as a Source Data file. A two-tailed, unpaired Student's t-test was used for comparison of M$^6_{JRS4}$, TB24 and TB33 experimental groups. For all other comparisons, a two-tailed, one-sample t-test was used to determine differences from the maximum resolution of the Etest assay (32 µg/ml).
**Supplementary Table 3.**

| Strain          | SXT MIC + THF (ng/cm²) |
|-----------------|------------------------|
|                 | 0         | 200     | 300     | 400     |
| TB08 (T71; dfrG<sup>+</sup>) | 0.125     | >32     | >32     | >32     |
| TB24 (A71)      | 0.047     | 0.50    | 0.75    | 0.75    |
| TB33 (A71)      | 0.032     | 0.38    | 0.38    | 0.38    |

Susceptibility of TB08, TB24 and TB33 to SXT in the presence of different concentrations of exogenous THF. Results are median values (µg/ml) of three independent experiments determined with Etest strips on MHF-Bm agar. Source data are provided as a Source Data file.
### Supplementary Table 4

| Strain                  | MIC (µg/ml) no THF | MIC (µg/ml) with THF | p value |
|-------------------------|--------------------|----------------------|---------|
|                         | SMX                | TMP                  | SXT     | SMX    | TMP    | SXT    |         |
| JRS4/vector             | 8.00 ± 0.00        | 0.273 ± 0.097        | 0.042 ± 0.009 | 7.33 ± 1.15 | 0.317 ± 0.110 | 0.034 ± 0.012 | 0.374 | 0.635 | 0.405 |
| JRS4/P23-thfT (T71)     | >1024              | 0.293 ± 0.075        | 0.058 ± 0.010 | >1024   | 0.337 ± 0.075 | 0.105 ± 0.035 | -     | 0.519 | 0.093 |
| JRS4/ thfT (T71)        | >1024              | 0.337 ± 0.075        | 0.058 ± 0.010 | >1024   | 0.377 ± 0.125 | 0.094 ± 0.031 | -     | 0.660 | 0.123 |
| JRS4/P23-thfT (A71)     | >1024              | 0.293 ± 0.075        | 0.068 ± 0.024 | >1024   | 0.397 ± 0.179 | 0.094 ± 0.031 | -     | 0.409 | 0.309 |
| JRS4/ thfT (A71)        | >1024              | 0.250 ± 0.000        | 0.058 ± 0.010 | >1024   | 0.337 ± 0.075 | 0.105 ± 0.035 | -     | 0.116 | 0.093 |

Mean, SD and p values for data in Fig. 4A. Values are mean ± SD for three biological replicates determined with Etest strips on MHF-Bm agar. A two-tailed, unpaired Student's t-test was used for comparison of groups.
### Supplementary Table 5

|          | MIC (µg/ml) no THF | MIC (µg/ml) with THF | p value |
|----------|--------------------|----------------------|---------|
|          | SMX    | TMP    | SXT    | SMX    | TMP    | SXT    | SMX    | TMP    | SXT    |
| NS5437   | 1.333 ± 0.289 | 0.250 ± 0.000 | 0.023 ± 0.000 | 1.667 ± 0.289 | 0.250 ± 0.000 | 0.023 ± 0.000 | 0.230 | 1.000 | 1.000 |
| NS5437::thfT | 3.667 ± 2.082 | 0.190 ± 0.000 | 0.023 ± 0.000 | >1024 | 0.190 ± 0.000 | 0.105 ± 0.035 | 3.600x10^-9 | 1.000 | 1.408x10^-3 |

Mean, SD and p values for data in Fig. 4C. Values are mean ± SD for three biological replicates determined with Etest strips on MHF-Bm agar. A two-tailed, one-sample t-test was used to determine differences from the maximum resolution of the SMX Etest assay (1024 µg/ml) for NS5437::thfT. A two-tailed, unpaired Student's t-test was used for comparison of NS5437 groups.
### Supplementary Table 6.

| Compound | Abbreviation | Supplier       | Cat#   | CAS#   |
|----------|--------------|----------------|--------|--------|
| Tetrahydrofolic acid \((\text{C}_{19}\text{H}_{23}\text{N}_{7}\text{O}_{6})\) | THF | Sigma Aldrich | T3125  | 135-16-0 |
| Dihydrofolic acid \((\text{C}_{19}\text{H}_{22}\text{N}_{7}\text{O}_{6})\) | DHF | Carbosynth    | FD170209 | 4033-27-6 |
| Folinic Acid \((\text{C}_{20}\text{H}_{23}\text{N}_{7}\text{O}_{7})\) | 5-formyl-THF | Carbosynth | FF156776  | 58-05-9 |
| 10-Formyl-5,6,7,8-tetrahydrofolic acid disodium \((\text{C}_{20}\text{H}_{21}\text{N}_{7}\text{Na}_{2}\text{O}_{7})\) | 10-formyl-THF | Carbosynth | FF168442  | 914800-65-0 |
| \((6\text{R,5S})\)-5,10-Methenyl-5,6,7,8-tetrahydrofolic acid \((\text{C}_{20}\text{H}_{21}\text{N}_{7}\text{O}_{6})\) | 5,10-me\(^{+}\)-THF | Sapphire Bioscience | FM11406  | 151533-22-1 |
| 5, 10-Methylene-5,6,7,8-tetrahydrofolic acid \((\text{C}_{20}\text{H}_{22}\text{N}_{7}\text{O}_{6})\) | 5,10-methyl-THF | Carbosynth | FM31365  | 3432-99-3 |
| 5-Methyltetrahydrofolic acid disodium salt \((\text{C}_{20}\text{H}_{21}\text{N}_{7}\text{Na}_{2}\text{O}_{6})\) | 5-methyl-THF | Carbosynth | FM31095  | 68792-52-9 |
| L-5-Methyltetrahydrofolate calcium \((\text{C}_{20}\text{H}_{22}\text{CaN}_{7}\text{O}_{6})\) | L-5-methyl-THF | Carbosynth | FM11406  | 151533-22-1 |

THF and related compounds used for metabolic rescue of GAS strains in the presence of antibiotics.
Supplementary Table 7

| Compound       | SMX MIC (µg/ml) NS5437 | NS5437::thfT | p value       |
|----------------|------------------------|--------------|---------------|
| none           | 1.333 ± 0.289          | 2.333 ± 0.577| 0.055         |
| THF            | 1.500 ± 0.000          | >1024        | 1.19x10^{-12}|
| DHF            | 0.917 ± 0.144          | >1024        | 1.19x10^{-12}|
| 5-formyl-THF   | 0.917 ± 0.144          | >1024        | 1.19x10^{-12}|
| 10-formyl-THF  | 0.917 ± 0.144          | >1024        | 1.19x10^{-12}|
| 5,10-me*THF   | 0.917 ± 0.144          | >1024        | 1.19x10^{-12}|
| 5,10-methylene-THF | 0.917 ± 0.144   | >1024        | 1.19x10^{-12}|
| 5-methyl-THF   | 1.167 ± 0.289          | 2.167 ± 0.764| 0.101         |
| L-5-methyl-THF | 0.833 ± 0.144          | 2.667 ± 0.577| 5.94x10^{-3}  |

Mean, SD and p values for data in Fig. 4A. Values are mean ± SD for three biological replicates determined with Etest strips on MHF-Bm agar. A two-tailed, one-sample t-test was used to determine differences from the maximum resolution of the SMX Etest assay (1024 µg/ml) for NS5437::thfT. For all other comparisons, a two-tailed, unpaired Student's t-test was used for comparison of groups.
## Supplementary Table 8

| Strain ID | emmST | MLST | Country | Year   | Site                          | thfT | dfrF | dfrG | Accession       |
|-----------|-------|------|---------|--------|-------------------------------|------|------|------|----------------|
| 7368_6_94 | 86    | ST4  | Australia | 2002  | joint / synovial fluid skin sore / abscess / burns / IV Site |      |      |      | GCA_900991495  |
| 7368_8_77 | 86    | ST4  | Australia | 1997  | skin sore / abscess / burns / IV Site |      |      |      | GCA_900992295  |
| 19944_7_58 | 117  | ST986 | Brazil   | 2003  |      |      |      |      | GCA_900985075  |
| SRR1104971 | 88   | ST971 | Canada   | 2010-2013 | ND |      |      |      | SRR1104971    |
| SRR1106008 | 74   | ST120 | Canada   | 2010-2013 | ND |      |      |      | SRR1106008    |
| 19944_6_141 | 104  | ST353 | India   | 2007-2010 | invasive |      |      |      | GCA_900983485  |
| 19944_6_145 | 80   | ST701 | India   | 2007-2010 | Throat |      |      |      | GCA_900983565  |
| 19944_6_146 | 75   | ST320 | India   | 2007-2010 | skin |      |      |      | GCA_900983525  |
| 19944_6_147 | 44   | ST987 | India   | 2007-2010 | Throat |      |      |      | GCA_900983515  |
| 19944_6_148 | 22   | ST360 | India   | 2007-2010 | skin |      |      |      | GCA_900983535  |
| 19944_6_154 | 44   | ST178 | India   | 2007-2010 | skin |      |      |      | GCA_900983615  |
| 19944_6_158 | 49   | ST354 | India   | 2007-2010 | ND |      |      |      | GCA_900983655  |
| 19944_6_160 | 104  | ST353 | India   | 2007-2010 | Throat |      |      |      | GCA_900983665  |
| 19944_7_4   | 75   | ST357 | India   | 2007-2010 | Throat |      |      |      | GCA_900984895  |
| 19944_7_5   | 49   | ST354 | India   | 2007-2010 | skin |      |      |      | GCA_900984955  |
| 19944_7_123 | 76   | ST378 | NZ      | 2013   | Throat |      |      |      | GCA_900984075  |
| 19944_7_129 | 76   | ST378 | NZ      | 2013   | Throat |      |      |      | GCA_900984115  |
| 19944_7_144 | 58   | ST1004 | NZ     | 2014   | Throat |      |      |      | GCA_900984275  |
| 19944_7_145 | 76   | ST378 | NZ      | 2013   | Throat |      |      |      | GCA_900984295  |
| 19944_8_45  | 76   | ST378 | NZ      | 2013   | Blood |      |      |      | GCA_900994755  |
| ERR1359618  | 58   | ST985 | UK      | 2014   | ND |      |      |      | ERR1359618     |
| ERR1359648  | 58   | ST985 | UK      | 2014   | ND |      |      |      | ERR1359648     |
| ERR1732468  | 11   | ST293 | UK      | 2014-2015 | ND |      |      |      | ERR1732468     |
| ERR1732551  | 74   | ST120 | UK      | 2014-2015 | ND |      |      |      | ERR1732551     |
| ERR1732614  | 49   | ST371 | UK      | 2014-2015 | ND |      |      |      | ERR1732614     |
| ERR1732992  | 76   | ST378 | UK      | 2014-2015 | ND |      |      |      | ERR1732992     |
| ERR1733243  | 76   | ST378 | UK      | 2014-2015 | ND |      |      |      | ERR1733243     |
| ERR1733400  | 218  | ST292 | UK      | 2014-2015 | ND |      |      |      | ERR1733400     |
| ERR1733501  | 49   | ST228 | UK      | 2014-2015 | ND |      |      |      | ERR1733501     |
| ERR1733622  | 49   | ST371 | UK      | 2014-2015 | ND |      |      |      | ERR1733622     |
| SRS2372037  | 82   | ST896 | USA     | 2015   | ND |      |      |      | SRS2372037     |
| SRS2372224  | 81   | ST909 | USA     | 2015   | ND |      |      |      | SRS2372224     |

Carriage of *thfT*, *dfrF* and *dfrG* by GAS. Genome sequences from reference 27. Presence (black) and absence (white) of *thfT*, *dfrF* and *dfrG* in genome sequences was determined by BLAST.
Supplementary Table 9.

| S. dysgalactiae   | Country | Year | Site     | thfT | drfF | drfG | Accession     |
|-------------------|---------|------|----------|------|------|------|---------------|
| (n=136)           |         |      |          |      |      |      |               |
| DY107             | China   | 2020 | Cow      |      |      |      | GCF_019856435 |
| WCHSDSE-1         | China   | 2013 | Throat   |      |      |      | GCF_001038445 |
| UT_4277_BB        | USA     |      | Sterile  |      |      |      | GCF_001682765 |
| DB31752-13        | Singapore | 2013 | Sterile  |      |      |      | GCF_009650255 |

Carriage of thfT, drfF and drfG by S. dysgalactiae. Refseq genome sequences downloaded from NCBI on 12 August 2022. Presence (black) and absence (white) of thfT, drfF and drfG in genome sequences was determined by BLAST.
Supplementary Table 10.

| RT  | m/z   | Formula         | Annotation       | VIP   | Higher |
|-----|-------|-----------------|-----------------|-------|--------|
| 9.58| 562.8210 | C54H89N7O18 |                 | 9.31045 | MH-Ox |
| 8.46| 326.7050 | C28H45N17O2 |                 | 8.88326 | MH-Ox |
| 9.40| 530.2970 | C26H43NO10 |                 | 7.69593 | MH-Ox |
| 7.96| 191.6260 | C18H31N5O4 |                 | 6.74514 | MH-Bm |

Positive

| RT  | m/z   | Formula         | Annotation       | VIP   | Higher |
|-----|-------|-----------------|-----------------|-------|--------|
| 8.10| 385.2440 | C18H32N4O5 |                 | 6.56549 | MH-Ox |
| 1.08| 136.0620 | C5H5N5   | Adenine         | 6.41266 | MH-Ox |
| 4.34| 284.0990 | C10H13N5O5 | Guanosine       | 6.33763 | MH-Bm |
| 9.32| 379.7080 | C34H47N17O4 |                 | 6.29898 | MH-Ox |
| 8.63| 342.2380 | C17H31N3O4 | Pro Ile Ile     | 6.28548 | MH-Bm |
| 8.17| 328.2230 | C16H29N3O4 | Pro Val Ile     | 6.19526 | MH-Bm |

Negative

| RT  | m/z   | Formula         | Annotation       | VIP   | Higher |
|-----|-------|-----------------|-----------------|-------|--------|
| 1.41| 111.0200 | C4H4N2O2   | Uracil          | 10.2991 | MH-Ox |
| 2.61| 487.1320 | C18H34O9P2S |                 | 9.50986 | MH-Bm |
| 4.34| 282.0850 | C10H13N5O5 | Guanosine       | 8.31936 | MH-Bm |
| 2.61| 243.0620 | C9H12N2O6  | Uridine         | 6.79399 | MH-Bm |
| 1.87| 128.0350 | C5H7NO3   | Pyrrolidonecarboxylic acid | 6.65050 | MH-Ox |
| 8.09| 243.1710 | C12H24N2O3 |                 | 5.33297 | MH-Bm |
| 8.78| 340.1880 | C16H27N3O5 |                 | 5.30046 | MH-Bm |
| 2.72| 457.1940 | C19H30N4O9 |                 | 5.26745 | MH-Bm |
| 1.42| 148.0440 | C5H11NO2S | L-Methionine    | 5.04665 | MH-Bm |
| 1.73| 191.0200 | C6H8O7   | Citric acid     | 4.74788 | MH-Ox |

Discriminating features identified under positive and negative ionisation modes following untargeted LC-MS profiling of MH-Bm and MH-Ox media.