Figure S1. Schematic figure of the *in vivo* passage of MS-H strain five times: Briefly, five 2-weeks-old specific-pathogen-free chickens were vaccinated by eyedrop with 50µL of MS-H. The birds were then euthanised at 4 weeks of age and the tracheas were removed. Tracheal washing was prepared by aspirating 5 ml of MB through the trachea for 10 times. The nasal turbinates were added to the tracheal washings and vortexed vigorously. Serial ten-fold dilutions of the mixtures were incubated at 33°C until late logarithmic phase. A total volume of 50µL from the lowest dilution was used for eyedrop administration to the next five chickens. The remaining culture from the lowest dilution of washing/nasal turbinates mixtures were transferred onto MA and incubated at 37°C for 10 days. This process was repeated through five chicken passages. A total number of 15 colonies (three per passage) were selected from MA plates and grown in 40 mL MB and incubated at 37°C until late logarithmic phase.
Figure S2. Schematic figure of the laboratory simulation of on-farm vaccination: A total of five four-week-old chickens were vaccinated with 0.1 mL dose of the MS-H vaccine, containing $10^{7.6}$ colour changing units (CCU) by eye drop (equivalent to the maximum release titre of $10^{9.1}$ CCU/mL). Swabs from upper, middle and lower trachea were taken from five chickens after 60 days post vaccination and inoculated immediately onto MA plates and incubated at 37°C for 7 days. A total number of 25 colonies (five per bird) were grown in 40 mL MB and incubated at 37°C until late logarithmic phase. The MS-H vaccine strain used in this experiment was also grown in 40 mL MB and incubated at 37°C until late logarithmic phase.
| Accession | Host species | Host type | Sample collection year | Age of host | Country of origin | Comment |
|-----------|--------------|-----------|------------------------|-------------|-------------------|---------|
| AB1 | chicken | | 1993 | 28 weeks | Australia | |
| AS2 | chicken | | 1993 | 11 weeks | Australia | |
| TS3 | chicken | | 1993 | 14 weeks | Australia | |
| TS4 | chicken | | 1994 | 28 weeks | Australia | |
| TS5 | chicken | | 1994 | 37 weeks | Australia | |
| TS6 | chicken | | 1994 | 55 weeks | Australia | |
| I45 | chicken | | 2014 | 59 weeks | Italy | Isolate IZSVE/2014/368-2* |
| I46 | chicken | | 2014 | 59 weeks | Italy | Isolate IZSVE/2014/368-6* |
| I47 | chicken | | 2014 | 59 weeks | Italy | Isolate IZSVE/2014/374-11* |
| I48 | chicken | | 2014 | 59 weeks | Italy | Isolate IZSVE/2014/374-12* |
| M152 | chicken | Broiler | 2015 | | Hungary | |
| M153 | chicken | Broiler | 2015 | | Hungary | |
| M155 | chicken | Broiler | 2015 | | Hungary | |
| M157 | chicken | Broiler | 2015 | | Hungary | |
| M163 | chicken | Broiler | 2015 | | Hungary | |
| M200 | chicken | Broiler | 2015 | 50 weeks | Hungary | |
| M214 | chicken | Layer | 2015 | 63 weeks | Hungary | |
| M215 | chicken | Layer | 2015 | 63 weeks | Hungary | |
| M235 | chicken | Breeder | 2015 | 47 weeks | Hungary | |
| M236 | chicken | | 2015 | 35 weeks | Hungary | |
| M239 | chicken | Breeder | 2015 | 63 weeks | Hungary | |
| M240 | chicken | Breeder | 2015 | 35 weeks | Hungary | |
| I01 | chicken | Breeder | 2015 | 60 weeks | Italy | |
| I02 | chicken | Breeder | 2015 | 39 weeks | Italy | |
| I03 | chicken | Breeder | 2015 | 42 weeks | Italy | |
| I04 | chicken | Breeder | 2015 | 18 weeks | Italy | |
| I05 | chicken | Breeder | 2015 | 18 weeks | Italy | |
| I06 | chicken | Breeder | 2015 | 44 weeks | Italy | |
| M254 | chicken | Layer | 2016 | 71 weeks | Hungary | |
| M272 | chicken | | 2016 | | Ukraine | |
| M289 | chicken | | 2016 | | Hungary | |
| M306 | turkey | | 2016 | | Hungary | off-label use under veterinary direction |
| M308 | turkey | | 2016 | | Hungary | off-label use under veterinary direction |
| I07 | chicken | Breeder | 2016 | 31 weeks | Italy | |
| I08 | chicken | Breeder | 2016 | 31 weeks | Italy | |
| Code | Type              | Year | Age   | Country      |
|------|-------------------|------|-------|--------------|
| I09  | chicken Breeder   | 2016 | 31 weeks | Italy       |
| I10  | chicken Breeder   | 2016 | 31 weeks | Italy       |
| I11  | chicken Breeder   | 2016 | 31 weeks | Italy       |
| I12  | chicken Breeder   | 2016 | 31 weeks | Italy       |
| I13  | chicken Breeder   | 2016 | 31 weeks | Italy       |
| I14  | chicken Breeder   | 2016 |       | Italy        |
| I15  | chicken Layer     | 2016 | 30 weeks | Italy       |
| I16  | chicken Breeder   | 2016 |       | Italy        |
| I17  | chicken Breeder   | 2016 |       | Italy        |
| I18  | chicken Breeder   | 2016 |       | Italy        |
| I23  | chicken Layer     | 2016 |       | Italy        |
| M376 | chicken Breeder   | 2017 | 16 weeks | Romania     |
| M378 | chicken Breeder   | 2017 | 17 weeks | Romania     |
| M379 | chicken Breeder   | 2017 | 15 weeks | Romania     |
| M380 | chicken Breeder   | 2017 | 13 weeks | Romania     |
| M381 | chicken Breeder   | 2017 | 22 weeks | Romania     |
| I19  | chicken Breeder   | 2017 | 27 weeks | Italy       |
| I20  | chicken Breeder   | 2017 |       | Italy        |
| I21  | chicken Layer     | 2017 |       | Italy        |
| I22  | chicken Broiler   | 2017 |       | Italy        |
| I24  | chicken Layer     | 2017 | 19 weeks | Italy       |
| I25  | chicken Layer     | 2017 |       | Italy        |
| I26  | chicken Layer     | 2017 | 40 weeks | Italy       |
| I27  | chicken Breeder   | 2017 | 40 weeks | Italy       |
| I28  | chicken Breeder   | 2017 | 40 weeks | Italy       |
| M510 | chicken Breeder   | 2018 |       | Slovakia     |
| M516 | chicken Broiler Breeder | 2018 | 26 weeks | India       |
| M517 | chicken Breeder   | 2018 | 23 weeks | India       |
| M528 | chicken Breeder   | 2018 |       | China        |
| M529 | chicken Breeder   | 2018 |       | China        |
| M542 | chicken Breeder   | 2018 | 16 weeks | India       |
| M544 | chicken Broiler Breeder | 2018 | 22 weeks | India       |
| M545 | chicken Broiler Breeder | 2018 | 22 weeks | India       |
| I29  | chicken Breeder   | 2018 | 8 weeks  | Italy       |
| I30  | chicken Breeder   | 2018 | 6 weeks  | Italy       |
| I31  | chicken Layer     | 2018 | 7 weeks  | Italy       |
| I32  | chicken Layer     | 2018 | 9 weeks  | Italy       |
| I33  | chicken Layer     | 2018 |       | Italy        |
| I34  | chicken Layer     | 2018 | 12 weeks | Italy       |
| I35  | chicken Layer     | 2018 |       | Italy        |
| I36  | chicken Layer     | 2018 | 22 weeks | Italy       |
| I37  | chicken Layer     | 2018 | 22 weeks | Italy       |
| I38  | chicken Breeder   | 2018 |       | Italy        |
| I39  | chicken Breeder   | 2018 |       | Italy        |
| Code  | Species     | Category     | Year | Age     | Country            |
|-------|-------------|--------------|------|---------|--------------------|
| M594  | chicken     |              | 2019 | 15 weeks| Romania            |
| M618  | chicken     | Layer        | 2019 | 50 weeks| Romania            |
| A-A   | chicken     |              | 2019 |         | Argentina          |
| A-B   | chicken     |              | 2019 |         | Argentina          |
| A-C   | chicken     |              | 2019 |         | Argentina          |
| A-D   | chicken     |              | 2019 |         | Argentina          |
| A-E   | chicken     |              | 2019 |         | Argentina          |
| A-F   | chicken     |              | 2019 |         | Argentina          |
| I41   | chicken     | Breeder      | 2019 |         | Italy              |
| I42   | chicken     | Breeder      | 2019 |         | Italy              |
| I43   | chicken     | Breeder      | 2019 | 32 weeks| Italy              |
| AQJ5A | chicken     |              | 2019 |         | Australia          |
| AQJ5B | chicken     |              | 2019 |         | Australia          |
| AQJ9B | chicken     |              | 2019 |         | Australia          |
| N01   | turkey      |              | 2019 |         | Netherlands        |
| N02   | turkey      |              | 2019 |         | Netherlands        |
| N03   | chicken     | Broiler Breeder | 2020 |         | Netherlands        |
| N04   | chicken     | Broiler Breeder | 2020 |         | Netherlands        |
| N05   | chicken     | Broiler Breeder | 2020 |         | Netherlands        |

*Isolates have been used in the following study: Moronato et al., 2018. Application of different laboratory techniques to monitor the behaviour of a *Mycoplasma synoviae* vaccine (MS-H) in broiler breeders. Vet. Res. 14:1-9.*
### Table S2. Details of clone preparation in this study

| Experiment type | Clone name            | Number of passages | Culture volume         | Number of selected colonies | Colony propagation volume |
|-----------------|-----------------------|--------------------|------------------------|----------------------------|----------------------------|
| **In vitro**    |                       |                    |                        |                            |                            |
|                 | Small-scale progeny   | 6                  | 10 mL                  | 5                          | 40 mL                      |
|                 | Large-scale progeny   | 6                  | commercial fermenter   | 6                          | 40 mL                      |
| **In vivo**     | Field reisolate       | 1                  | NA                     | 98                         | Pure culture was provided  |
|                 | Controlled reisolate  | 1                  | NA                     | 25                         | 40 mL                      |
|                 | Passaged reisolates   | 5                  | NA                     | 15                         | 40 mL                      |
| Locus-tag     | Product                                                                 | Number of genomes containing variation(s) (98) |
|---------------|--------------------------------------------------------------------------|-----------------------------------------------|
| MSH_RS01740   | ABC transporter, OppF                                                    | 62                                           |
| MSH_RS00965   | GTPase, ObgE                                                            | 54                                           |
| MSH_RS01365   | Glyceraldehyde-3-phosphate dehydrogenase, GAPDH                         | 13                                           |
| MSH_RS00320   | DNA topoisomerase IV subunit A, ParC                                     | 14                                           |
| MSH_RS01430   | P80 family lipoprotein                                                  | 12                                           |
| MSH_RS02465   | DNA-directed RNA polymerase subunit beta                                | 12                                           |
| MSH_RS00575   | Sugar ABC transporter                                                   | 10                                           |
| MSH_RS03140   | M42 family metallopeptidase                                             | 8                                            |
| MSH_RS00255   | Hypothetical protein                                                    | 7                                            |
| MSH_RS01685   | P80 family lipoprotein                                                  | 7                                            |
| MSH_RS03070   | Type IIA DNA topoisomerase subunit B                                    | 7                                            |
| MSH_RS02015   | Thymidylate synthase                                                    | 6                                            |
| MSH_RS02845   | Hypothetical protein                                                    | 5                                            |
| MSH_RS03170   | Lysine--tRNA ligase                                                     | 3                                            |
| MSH_RS02670   | Phosphoenolpyruvate--protein phosphotransferase                         | 3                                            |
| MSH_RS03065   | Serine--tRNA ligase                                                     | 3                                            |
| MSH_RS02470   | DNA-directed RNA polymerase subunit beta                                | 3                                            |
| MSH_RS02775   | Hypothetical protein                                                    | 3                                            |
| MSH_RS01875   | SGNH/GDSL hydrolase family protein                                      | 2                                            |
| MSH_RS01920   | Transcription elongation factor GreA                                    | 2                                            |
| MSH_RS03630   | Translation initiation factor IF-2                                      | 2                                            |
| MSH_RS02945   | tRNA pseudouridine synthase B                                           | 2                                            |
| MSH_RS02350   | tRNA-Asp                                                                | 2                                            |
| MSH_RS02560   | ECF transporter S component                                              | 2                                            |
| **MSH_RS01000** | **P80 family protein**                                               | **2**                                        |
| MSH_RS01195   | Hypothetical protein                                                    | 2                                            |
| MSH_RS01640   | Hypothetical protein                                                    | 2                                            |
| MSH_RS00140   | Glycerophosphodiester phosphodiesterase                                | 2                                            |
| MSH_RS02780   | UvrD-helicase domain-containing protein                                 | 2                                            |
| MSH_RS02980   | Cation-translocating P-type ATPase                                     | 2                                            |
| MSH_RS03180   | ABC transporter permease                                                | 2                                            |
| MSH_RS00390   | Putative immunoglobulin-blocking virulence protein                      | 2                                            |
| Accession     | Description                                             | Count |
|--------------|---------------------------------------------------------|-------|
| MSH_RS01090  | PDxFFG protein                                          | 2     |
| MSH_RS01715  | Cell division protein FtsZ                              | 2     |
| MSH_RS02600  | ABC transporter ATP-binding protein                     | 2     |
| MSH_RS03505  | DAK2 domain-containing protein                          | 2     |
| MSH_RS03580  | Restriction endonuclease subunit S                      | 2     |
| MSH_RS03610  | PTS ascorbate transporter subunit IIC                   | 2     |
| MSH_RS03360  | Hypothetical protein                                    | 2     |
Table S4. Effect of amino acid substitution on stability and solvent accessibility of the proteins coded by mutation-prone regions

| Product | Template code used for modelling | Crystal structure source | Global model quality estimation (GMQE) | Sequence identity | Variation(s) | Solvent accessibility\(a\) (%) | Predicted pseudo ΔΔG\(b\) (kcal/mol) |
|---------|---------------------------------|-------------------------|----------------------------------------|-------------------|--------------|--------------------------------|----------------------------------|
| GAPDH   | 7jwk.1.A                        | *Mycoplasma genitalium* | 0.84                                   | 63.72%            | Lys306Arg    | +30.2 to +38                    | +0.13                            |
|         |                                 |                         |                                        |                   | Arg123Gly    | 21.2 to 17.2                    | -0.02                            |
|         |                                 |                         |                                        |                   | Ala210Val    | 0.4 to 0.4                      | 0.57                             |
| ObgE    | 1lnz.2.A                        | *Bacillus subtilis*     | 0.52                                   | 48.18%            | Arg123Gly    | 21.2 to 17.2                    | -0.02                            |
|         |                                 |                         |                                        |                   | Ala210Val    | 0.4 to 0.4                      | 0.57                             |
| ParC    | 2nov.2.A                        | *Streptococcus pneumoniae* | 0.39                                   | 47%               | Thr85Ile     | 101.4 to 99.6                   | 1.07                             |
|         |                                 |                         |                                        |                   | Asp89Asn     | 59.9 to 5.3                     | -0.94                            |

\(a\) Solvent accessibility of amino acid side chain was from reference (MS-H) to the variant; values less than 17% show inaccessible/buried side chain, values between 17 to 43% show partially accessible side change and values more than 43% is accessible.

\(b\) Delta G (ΔΔG) is the change in Gibbs free energy between the folded and unfolded states (ΔGfolding) when a point mutation is present; A negative ΔΔG value corresponds to mutation predicted to be destabilising the protein whereas a positive ΔΔG value corresponds to mutation predicted to be stabilising the protein.

Note: only proteins with an available and acceptable quality and identity template were assessed.