Material and methods

Fly stocks and *S. poulsonii* extraction

We used wild-type *Oregon-R* (*OR^R*) fly stock harboring MSRO *Spiroplasma*, a strain which naturally infects *D. melanogaster,* (20). As *Spiroplasma poulsonii* is temperature sensitive (21), all flies were maintained at 25°C. As *Spiroplasma poulsonii* are still uncultivable, all experiments were performed on freshly extracted *Spiroplasma poulsonii* hemolymph. Pure *Drosophila* hemolymph was collected from flies individually using a Drummond™ Nanoject and pulled in capillary needle (9). 20 to 25 flies were used per sample. *S. citri* was cultivated as described (22).

Antibiotic injection experiments

20 one-week-old *Spiroplasma* infected females were injected in lateral thorax with 9.6nl of different solutions using a Drummond™ Nanoject: PBS, DMSO, ethanol, PC190723 (50mM, Merck Millipore), ciprofloxacin (20µg/ml, Sigma Aldrich, St. Louis, MO, USA) or penicillin (2.10^5 U/ml, Sigma Aldrich, St. Louis, MO, USA). Electron microscopy observations, and evaluation of fluorescence per baterium were performed 4 days and one week after injection. Evaluation of bacterial loading was performed 1 week after injection.

Examination of Spiroplasma conformation by fluorescence microscope and quantification

To observe *Spiroplasma* in fly hemolymph, flies were dissected on microscope slides in 5 µl PBS containing 0.02 mM SYTO9 (Invitrogen, Carlsbad, CA, USA). Slides were then mounted and observed on an Axioimager Z1 (Zeiss, Oberkochen, Germany). Images were captured with an Axiocam MRn camera and Axiovision software. To determine the absolute number of bacteria per fly, we extracted hemolymph from 20 infected flies homogenized in 20 µl of PBS. We performed 4 serial dilutions followed by a SYTO9 treatment. *Spiroplasma* cell numbers were counted by fluorescence microcopy as described above. To estimate the amount of DNA per bacterium, the same diluted hemolymph extracts were suspended in 20 µl of water, heated at 95°C for 20 min to release DNA in buffer and

34 centrifuged at 13,000 rpm for 20 min to remove debris. *DNA* was then determined
35 using a Roche Lightcycler (LC) 2.0 detection system. For each 10 µl qPCR reaction, 5
36 µl of supernatant was mixed with 5 µl of LightCycler® 480 SYBR Green I Master mix
37 according manufacturer instructions (Roche). Spiroplasma dnaA gene primers used
38 were DnaA109F 5'-TTAAGAGCAGTTTCAAAATCGGG-3′ and DnaA246R 5′-
39 TGAAAAAAACAAACAAATTGTTATTACTTC-3′. The amount of DNA per
40 bacterium was obtained by the ration between the quantity of DNA as measured by
41 qPCR and the number of bacteria.
42
43 ***Scanning electron microscopy (SEM)***
44 Drops of cells contained in 50µl of PBS for *S. poulsonii* extracted from
45 hemolymph, or 50µl of SP4 medium for *S. citri* were spotted onto a silicon wafer.
46 These were left for 15 minutes before being fixed for 1 hour with 1.25%
47 glutaraldehyde and 1% tannic acid in 0.1 M phosphate buffer, pH 7.4. The wafers
48 were then washed in phosphate buffered saline, fixed again in 1.0 % osmium tetroxide
49 in the same buffer for 30 minutes, and then dehydrated in a graded alcohol series,
50 being drying by passing them through the critical point of carbon dioxide (Leica
51 Microsystems CPD300). The samples were then coated with a 2nm layer of osmium
52 using an osmium plasma coater (Filgen OPC60).
53 Scanning electron microscopy images were taken with a field emission
54 scanning electron microscope (Merlin, Zeiss NTS, Germany) using an acceleration
55 voltage of 2kV and the in-lens secondary electron detector.
56
57 ***Transmission electron microscopy (TEM)***
58 Cells were preserved for transmission electron microscopy using high
59 pressure freezing and freeze substitution. This required loading the cells in the 3 mm
60 aluminium carrier and freezing them with a high-pressure freezer (Leica HPM100,
61 Leica Microsystems). These samples were then placed into tubes, held in liquid
62 nitrogen, containing frozen acetone with 1 % osmium tetroxide, 0.5% uranyl acetate,
63 and 5% water. The tubes were then left overnight inside an ice bucket containing dry
64 ice, agitating gently on a shaker table. The following day the dry ice was removed and
65 the ice bucket and sample tubes allowed to warm to 0°C over the next 2 hours. At this
66 point the acetone was replaced, three times, with fresh acetone at 5 ° C, and then

67 embedded in increasing concentrations of epon resin, and then placed into molds and
68 cured for 48 hours at 65° C.
69 Thin (50 nm) sections of the embedded cells were cut using a diamond knife
70 and ultramicrotome (Leica UC7). Sections were collected on single slot copper grids
71 with a formvar support film, and then stained with lead citrate and uranyl acetate.
72 These were imaged inside a transmission electron microscope at 80 kV (Tecnai Spirit,
73 FEI Company), using a CCD camera (Eagle, FEI Company).
74 ***Cryo immuno electron microscopy***
75 For immuno-electron microscopy, cells were chemically fixed with a
76 buffered mix of 2% paraformaldehyde and 0.1% glutaraldehyde, and embedded in
77 12% gelatin. Small cubes (1 mm width) were then cut with a razor blade and then
78 infiltrated overnight in 2.3M sucrose before freezing in liquid nitrogen. These were
79 then sectioned at 100nm using an ultramicrotome (Leica UC7/FCS, Leica
80 Microsystems, Vienna) set at 100°C. Sections were collected on formvar coated grids
81 carrying a drop of methylcellulose/sucrose at room temperature and then processed
82 for immunogold labeling with polyclonal antibody anti-FtsZ (rabbit, Agrisera).
83

84 **Figure Legends**

85

86 **Figure 1. Presence of Y-shaped *S. poulsonii* in *Drosophila* hemolymph**
87 **samples**

88 **(A, B)** Fluorescent microscopy images showing SYTO-9 stained *S. poulsonii*
89 from freshly extracted *Drosophila* hemolymph of 1 week-old flies. Bars, 1 µm. (**C-F)**
90 SEM of *S. poulsonii* extracted from 1 week-old *Spiroplasma* infected females. *S.*
91 *poulsonii* can be found as one elongated body (C) or with a Y-shape conformation (D-
92 F) with variation in arms length. Bars, 1µm. (**G)** TEM microscopy of *S. poulsonii*
93 from freshly extracted *Drosophila* hemolymph. White arrowhead shows branching.
94 Bar, 1 µm. (**H)** Immunogold labeling pattern of FtsZ protein with an anti-FtsZ
95 antibody. Y-shaped bacteria have two FtsZ protein aggregates. Bar, 200nm. (**I, J)**
96 SEM microscopy of *in vitro* cultured *S. citri*. *S. citri* can be found as one elongated
97 body (I) or with an Y-shaped conformation (J). Bars, 1 µm. The presence of
98 aggregates might be due to proteins enrichment in the medium used to cultivate *S.*
99 *citri*.

100

101 **Figure 2. Impacts of various antibiotics on Y-shaped form Spiroplasma**

102 **(A-E)** SEM of *S. poulsonii* extracted from *Spiroplasma* infected females
103 injected with PBS (A), DMSO (B), ethanol (C), PC190723 (D) or ciprofloxacin (E).
104 One-week-old females were injected with molecules, and hemolymph was extracted
105 after 4 days. Bars, 500nm. (**F)** Quantification of normal and abnormal Y-shape *S.*
106 *poulsonii* by SEM in hemolymph samples collected from treated flies. p=0.05. Shown
107 is the mean ± SD of data pooled from three independent experiments with 100
108 bacteria for each count. 20 flies were used to extract fresh hemolymph for each
109 experiment. (**G)** Evaluation of bacterial count per fly following each treatment.
110 *Spiroplasma* were counted one week after injection. p=0.05. Shown is the mean ± SD
111 of data pooled from three independent experiments with 20 flies for each count. (**H)**
112 Quantification of fluorescence amount per bacteria. DNA fluorescence per
113 *Spiroplasma* was measured 4 days after injection. p=0.05. Shown is the mean ± SD of
114 data pooled from three independent experiments with 20 flies tested for each count.

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