Electronic Supplementary Information

The nature of inherent bactericidal activity: insights from the nanotopology of three species of dragonfly

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S-1 Additional Materials and methods

Bacterial strains, growth and sample preparation: Prior to each bacterial attachment experiment bacterial cultures were refreshed on nutrient agar from stocks (Oxoid, Basingstoke, Hampshire, UK). Fresh bacterial suspensions were grown overnight at 37 °C in 5 mL of nutrient broth (Oxoid, Basingstoke, Hampshire, UK). Bacterial cells were collected at the logarithmic stage of growth and the suspensions were adjusted to $OD_{600} = 0.3$ as previously described[1]. The insect wings, mounted on circular discs, were immersed in 5 mL of the bacterial suspension for incubation intervals of 1 hour, 3 hours or 18 hours.

To achieve spore formation and growth prior to incubation an aliquot from the vegetative cell suspension of *B. subtilis* NCIMB 3610^T was spread on to the nutrient agar (Oxoid) plate and incubated at 37 °C for 7 days in depletion of oxygen. Sporulation (>95 %) was observed microscopically on glass slides by staining spores with malachite green and counter-staining healthy cells with safranin[2] Spores were then suspended in phosphate buffered saline (PBS) solution, washed at 13000 rpm for 5 minutes and re-suspended. Before incubation on the insect wings, spore suspension in PBS were adjusted to $OD_{600} = 0.1$. The insect wings were then incubated with spore suspension for 1, 3 and 18 hours attachment experiments.

Wing surface composition: X-ray photoelectron spectroscopy (XPS) was performed using a Kratos Axis Ultra DLD X-ray photoelectron spectrometer (Kratos Analytical Ltd., UK) equipped with a monochromatic X-ray source (Al Kα, $h\upsilon$ = 1486.6 eV) operating at 150 W. The spectrometer energy scale was calibrated using the Au $4f_{7/2}$ photoelectron peak at binding energy (BE) of 83.98 eV. Samples were flooded with low-energy electrons during the analysis to counteract surface charging. The hydrocarbon component of the C 1s peak (binding energy 285.0 eV) was used as a reference for charge correction. The elements

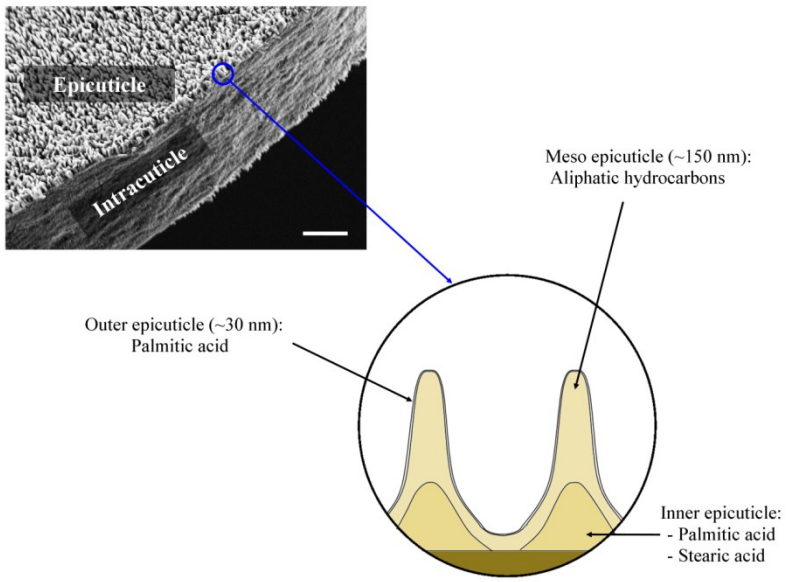
present on the surfaces were determined from survey spectra in the range 0-1400 eV at an interval and pass energy of 1 eV and 160 eV respectively. The relative atomic concentration of elements detected by XPS was quantified on the basis of the peak area in the survey spectra using sensitivity factors for the Kratos instrument. High resolution scans were performed across each of the C 1s, O 1s and Si 2p peaks, which were then fitted with Gaussian-Lorentzian components after the removal of a linear background signal (Kratos Vision II software).

Cell viability: A mixture of SYTO® 9 and propidium iodide fluorescent dyes (Molecular Probes™, Invitrogen, Grand Island, NY, USA) was used in the BacLight™ Bacterial Viability test. SYTO® 9 permeated both intact and damaged membranes of the cells, binding to nucleic acids and fluorescing green when excited by a 485 nm wavelength laser. While propidium iodide alone entered only cells with significant membrane damage, which are considered to be non-viable, and binds with higher affinity to nucleic acids than SYTO® 9.

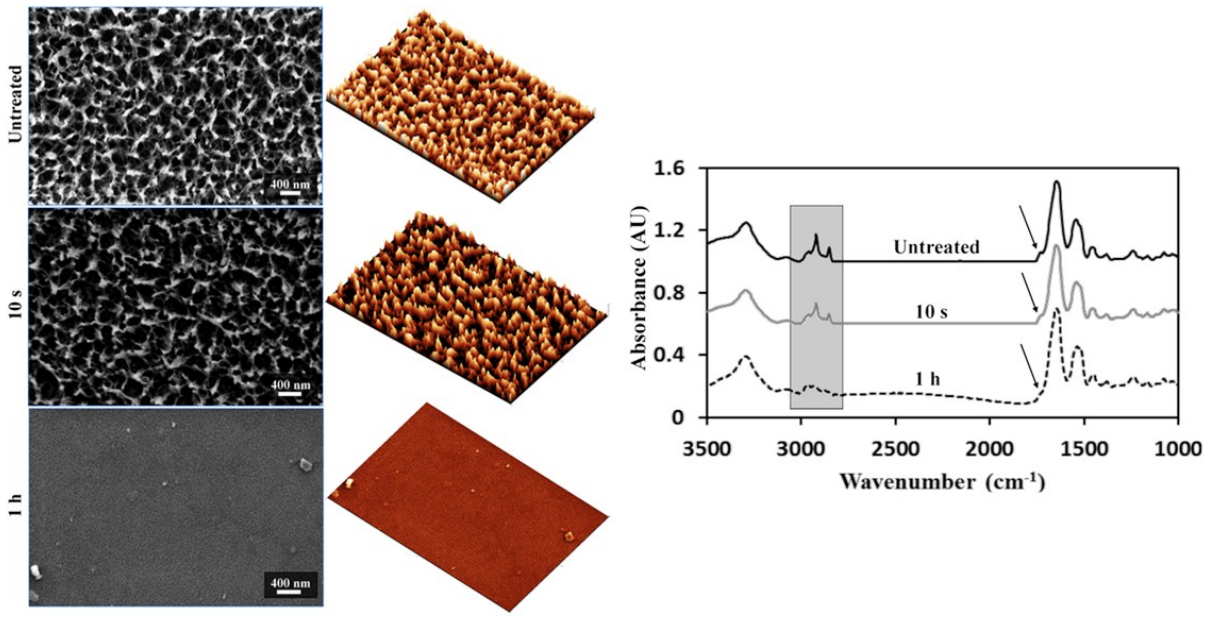
Bacterial viability assays utilized the re-suspended cells diluted to 1:10 and incubated in 3.5 cm diameter wells in triplicate with each well containing a 1 cm^2 area substratum wing sample. The cell suspensions were then sampled (100 μL) at discrete time intervals (3 hours and 18 hours), serially diluted 1:10, and each dilution spread on three nutrient agar plates. Resulting colonies were then counted, and the number of colony forming units per mL was calculated. The number of colony forming units was assumed to be equivalent to the number of live cells in suspension.

S-2 Dragonfly wing surface composition – nanostructure relationship

Nanostructures covering the surface of dragonfly *Hemianax papuensis* wings have been shown to be composed primarily of aliphatic hydrocarbons, with an outer layer composed largely of fatty acids arranged as a three layer epicuticle sequence (indicated by synchrotron m-FTIR, XPS and extract GC-MS) as illustrated below, and which have been shown not to contain carbohydrates or proteins (Lapointe et al. 2004, Agric For Entomol). Below this lies the proteinaceous chitin intracuticle layer as indicated by transmission mode m-FTIR (Ivanova et al., 2013, PLOSOne, Hasan & Ivanova et al., 2012, Langmuir).



Micrographs and 3D images of dragonfly wings prior to, and after 10 s and 1 h of chloroform extraction revealed the underlying chitin intracuticle sub-layer (1 h) while the corresponding transmission m-FTIR scans in transmission mode shows the continuing bare chitin layer.



S-3 Bactericidal activity of wing surface composition – nanostructure relationship

The origin of the bactericidal activity was shown to be related directly to nanomechanical stresses induced by the epicuticular nanotopology (Ivanova et al., 2013, Nature Comm.) where it was shown to be independent of specific chemical functionality by gold coating respective wing surfaces. Below it can be seen that the nanotopology of the wings is sufficient to generate a potent bactericidal effect independent of the chemical composition when coated with thin (10 nm) layers of gold, to eliminate effects of the wing chemistry, as seen for *P. aeruginosa* (left) and *S. aureus* (right) cells killed by the gold-sputtered *D. bipuncata* dragonfly wing surface.

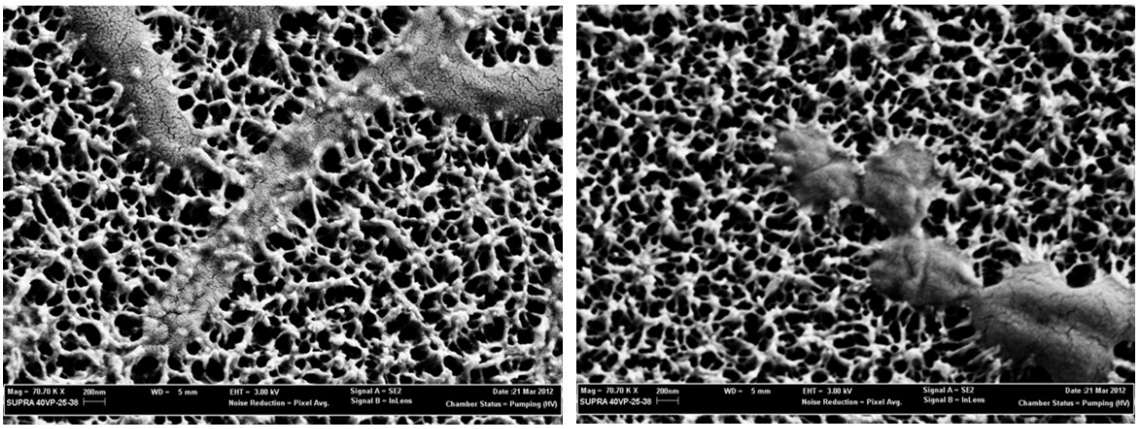


Fig. S-1. (A) *Hemianax papuensis*, (B) *Austroaeschna multipunctata* and (C) *Diplacodes bipunctata* dragonflies (not to scale). Reproduced with permissions from Encyclopedia of Life (eol.org, license agreements can be found at **http://creativecommons.org/licenses/by-nc-sa/2.0/** and **http://creativecommons.org/licenses/by-nc/2.0/**.

Table S-1. Characteristics of dragonfy species

	Wing span (cm)	Body length (cm)	Lava (cm)
H. papuensis	11.0	7.0	4.5 - 4.9
A. multipunctata	8.0	6.0	3.5 - 3.8
D. bipunctata	5.5	3.5	1.1 - 1.4

* Theischinger, G; Hawking, J (2006). *The Complete Field Guide to Dragonflies of Australia*. Collingwood Vic.: CSIRO Publishing. p. 276. ISBN 978 0 64309 073 6.

Table S-2. Wetting characteristics of dragonfly wings together with overall killing efficiency

Depth (nm)	Contact angle θ_{water}	θ_{water} hysteresis	Overall killing efficiency 10^4 cells cm^{-2} min^{-1}	
			cells	spores
H. papuensis	161.8 ± 1.9°	2.7°	5.7	4
A. multipunctata	157.5 ± 1.7	4.3	7.3	7
D. bipunctata	151.7 ± 2.8	2.7	29.7	10

Table S-3. Bearing analysis of dragonfly wings. The bearing ratios of each wing at 50 nm intervals, indicating the proportion of the projected area occupied by the surface at the given depths. Bearing data calculated from 2.5 μm × 2.5 μm AFM scans.

	Bearing ratio (%)		
Depth (nm)	*H. papuensis*	*A. multipunctata*	*D. bipunctata*
50	0.166702	0.867081	0.129318
100	2.01797	9.81712	3.79219
150	13.0581	36.1801	27.0264
200	42.9798	70.0306	66.6794
250	73.4138	88.1317	90.8859
300	90.2287	97.0276	98.9086
350	97.9343	99.7215	99.9382
400	99.6281	99.9996	
450	99.9962		

Table S-4. Forces imposed at the 3-phase contact line

	Depth (h) where downward tensional force begins (F=0)	Maximum downward tensional force F x 10^9 N	Gradient of downward tensional force at F=0	Maximum bactericidal efficiency $10^4 cm^{-2}\ min^{-1}$
H. papuensis	245	5.0	1.63	5
A. multipunctata	190	5.2	1.54	11
D. bipunctata	210	6.5	2.51	46

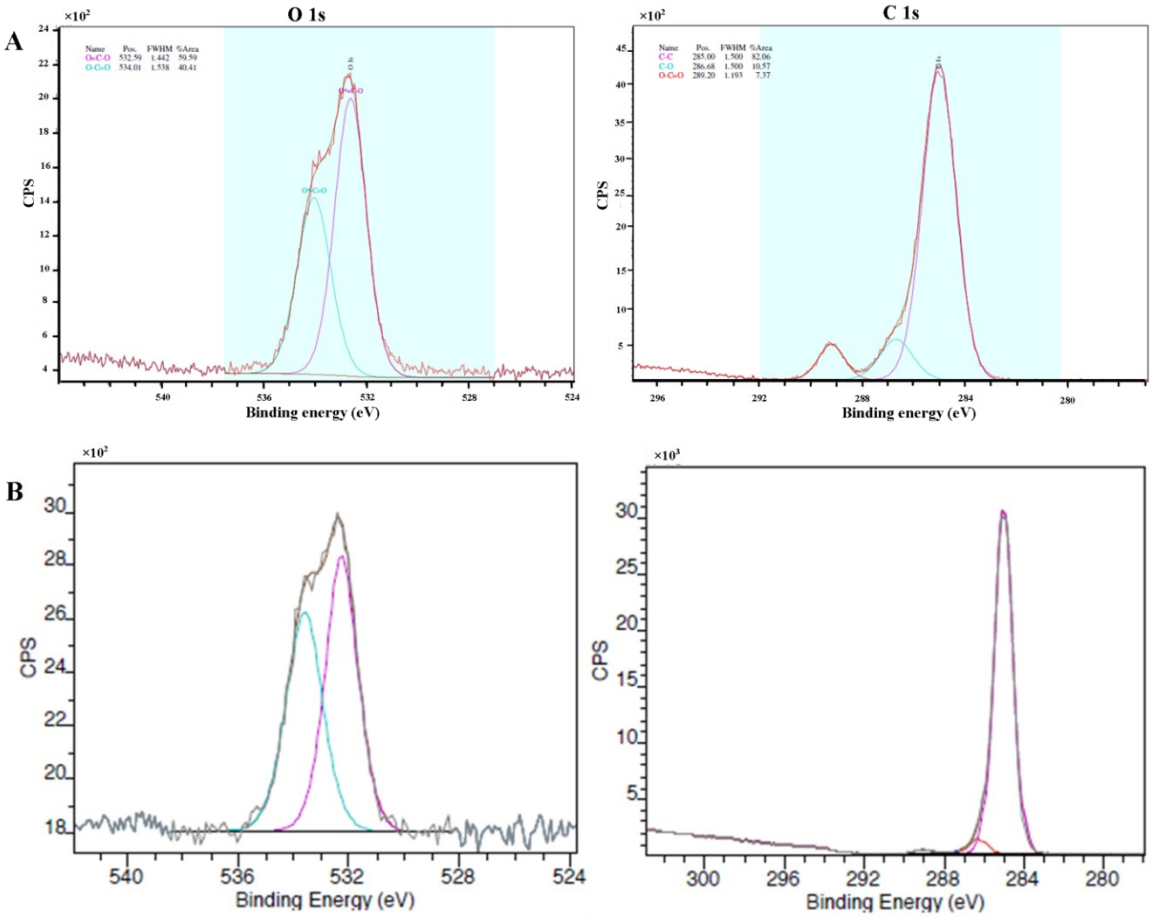


Fig. S-2. High resolution XPS spectra of (*A*) *H. papuensis* and (*B*) *D. bipunctata* dragonfly wings. High-resolution scans were performed in approximately 20 eV intervals across the O_{1s} and C_{1s} peaks. C 1s revealed the presented of carbon saturated and C-COO, whereas there are only one major oxygen peak. This can be observed at spectra of both species indicated their similarities in surface chemistry.

Additional references

1. S. L. Lapointe , W. B. Hunter, R. T. Alessandro, *Agriculture and Forest Entomology*, 2004, 6, 251–257.

2. J. W. Bartholomew and T. Mittwer, *Biotechnic & Histochemistry*, 1950, 25, 153-156.

3. E. P. Ivanova, J. Hasan, H. K. Webb, V. K. Truong, G. S. Watson, J. A. Watson, V.A. Baulin, S. Pogodin, J. Y. Wang, M. J. Tobin, C. Löbbe and R. J. Crawford, *Small*, 2012, 8, 2489-2494.