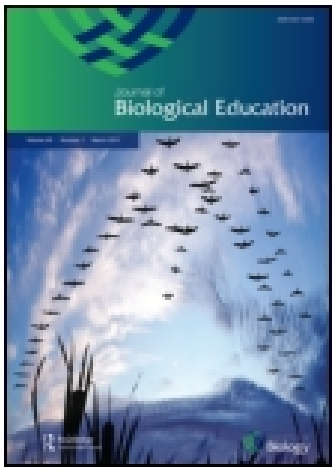

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Evolution in a test tube: rise of the Wrinkly Spreaders

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Practical

Evolution in a test tube: rise of the Wrinkly Spreaders

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Understanding evolutionary mechanisms is fundamental to a balanced biological education, yet practical demonstrations are rarely considered. In this paper we describe a bacterial liquid microcosm which can be used to demonstrate aspects of evolution, namely adaptive radiation, niche colonisation and competitive fitness. In microcosms inoculated with *Pseudomonas fluorescens* SBW25, evolved mutants such as the Wrinkly Spreader (WS) rapidly arise to form biofilms covering the air–liquid (A–L) interface. WS are readily isolated due to a distinctive colony morphology and reach ~30% of the population within five days. When re-inoculated into static microcosms, WS preferentially colonises the A–L interface by producing a biofilm, demonstrating a niche preference distinct from the ancestral SBW25 which grows throughout the liquid column. This ability provides the WS with a ~2.5× competitive fitness advantage over the non-biofilm forming ancestral SBW25. However, WS and SBW25 have similar fitness in shaken microcosms where biofilms cannot form. These practical demonstrations of WS evolution, suitable for secondary or tertiary-level classes, can be linked with a literature-based review of the underlying molecular biology of the WS phenotype to provide a true exemplar of the modern evolutionary synthesis, the current paradigm in evolutionary biology.

Keywords: bacteria; evolution; fitness; microcosms; mutation; niche

Introduction

Evolution is often described as a process which generally occurs over thousands of generations and millions of years. Key elements to the process of evolution are adaptive radiation, niche preference and fitness advantage. Adaptive radiation is the rapid speciation of a single or a few species to fill many ecological niches and is driven by mutation and natural selection. The success of a novel adaptive mutant can be measured in terms of fitness compared to the ancestor or co-evolved siblings. Adaptive mutants sometimes display a different niche preference compared to their competitors, and the ability to colonise a new environment is often the mechanism which provides the fitness advantage. While adaptive radiation is typically illustrated by reference to Darwin's finches of the Galápagos Islands or the Cichlid fish of the African Great Lakes, it also occurs in bacteria resulting in enormous diversity and the colonisation of almost all habitats.

Adaptive radiation in bacteria can be studied using artificial microcosms, and, due to the short generation time, rapid growth rate and high population densities achievable with artificial growth media, experiments

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involving bacteria can be completed in remarkably short periods of time (reviewed by Rainey et al. 2000). One such example uses the soil and plant-associated, non-pathogenic bacterium *Pseudomonas fluorescens* SBW25 grown in vials containing King's B liquid medium. The bacterium can be incubated over three to five days with shaking, or statically with no disturbance at all (this was first reported by Rainey and Travisano 1998). The rapid population explosion of SBW25 in these microcosms results in radiation, i.e. the accumulation of mutants which are often referred to as novel genotypes. In these conditions, the random mutation rate of SBW25 is $\sim 10^{-7}$ per generation. While some types of mutation may be lethal or have no impact (i.e. are silent), other mutations in SBW25 produce colonies with altered morphology and are referred to as 'morphs' (an abbreviation of 'morphotypes'). These novel morphs are readily differentiated from the smooth, rounded colonies of the ancestral, wild-type SBW25 on King's B agar plates (Figure 1). One particular colony morph, the Wrinkly Spreader (WS), characterised by a flat and wrinkled morphology, was found to colonise the air–liquid (A–L) interface of static microcosms by producing a cellulose-based biofilm. In contrast, Smooth (SM) morphs including wild-type SBW25 colonised the liquid column and Fuzzy Spreaders (FS) the bottom of the microcosm. The WS, SM and FS morphs are all genetically derived from the ancestral SBW25 inoculum, and the diversification of SBW25 into these novel genotypes is highly reproducible.

The ability of the WS to produce a biofilm at the A–L interface of static microcosms is an example of altered niche preference, and the enhanced ecological success of WS can be quantified by simple fitness assays. Success in this case is a faster rate of growth than genotypes located deeper in the microcosm where low oxygen levels limits growth (there is also a cost associated to WS biofilm formation: cellulose is an energy-rich glucose-based polymer, this energy could be otherwise used for growth). The mutation responsible for the WS is known and the underlying molecular biology (briefly outlined in Figure 2) is accessible via Spiers and Rainey (2005), Bantinaki et al. (2007) and references therein.

The combination of our ecological and molecular understanding of the WS in static liquid microcosms makes this experimental demonstration of 'evolution in a test tube' virtually unique and an ideal exemplar of the modern evolutionary synthesis. In this paper we

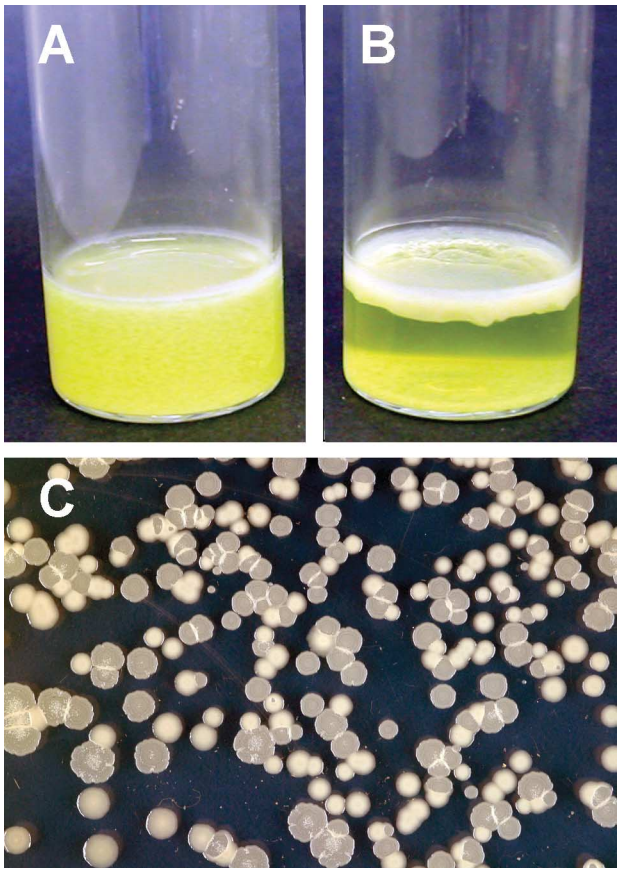

Figure 1. Adaptive radiation in static microcosms gives rise to the Wrinkly Spreader with a new niche preference

Notes: Shown are two static King's B liquid medium microcosms with (A) wild-type 'ancestral' SBW25 which grows throughout the liquid column, and (B) the evolved Wrinkly Spreader (WS) which shows a preference for the air–liquid (A–L) interface which it colonises by the production of a robust biofilm. An example of the diversity of colony morphologies seen in evolving SBW25 populations is given in (C). Photographs taken by Radoslaw Pajor.

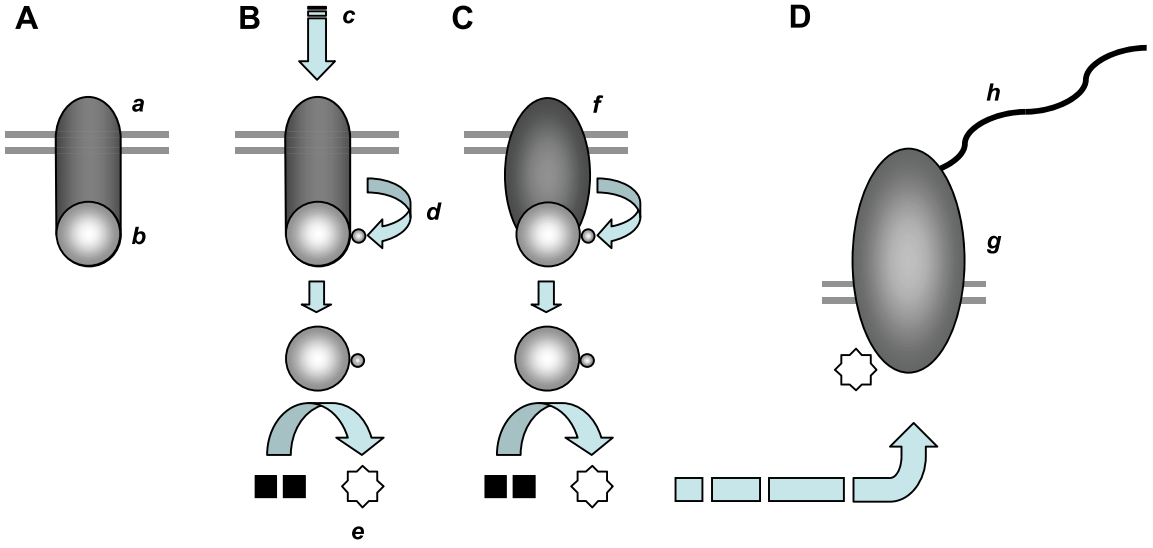

Figure 2. Activation of the Wrinkly Spreader. A schematic of the activation pathway in the Wrinkly Spreader (WS) is shown

Notes. (A) Under normal circumstances the Wsp chemosensory complex (a) and associated WspR regulator (b) are inactive. (B) When the appropriate environmental signal (c) is received, the Wsp complex activates WspR by phosphorylation (d) which produces the secondary messenger cyclic-*di*-GMP (e) from GMP. (C) In the WS, a mutation in the Wsp complex (f) leads to the constant activation of WspR and production of cyclic-*di*-GMP. (D) Cyclic-*di*-GMP activates cellulose synthase (g) to produce cellulose (h), a central element of the WS phenotype which includes the WS biofilm and wrinkled colony morphology. The environmental signal received by the Wsp complex is still unknown. Figure after Bantinaki et al. (2007).

present three experiments illustrating adaptive radiation, niche colonisation and competitive fitness based on the SBW25 experimental microcosm for use in secondary schools and tertiary-level practical classes.

Methodology

Bacteria and culturing conditions

Wild-type *Pseudomonas fluorescens* SBW25 can be obtained by contacting AS. WS mutants are obtained from the adaptive radiation experiment described below. Bacteria were grown in King's B (KB) medium (10 g glycerol, 1.5 g K_2HO_4, 1.5 g $MgSO_4.7H_2O$ and 20 g Proteose peptone No. 3 (Becton, Dickinson and Company, Cat. BD 211693) made up to 1 litre with deionised water). KB agar plates contained 12 g agar/l. For long-term storage, strains are kept at –80°C in KB with 15% (v/v) glycerol. Microcosms are sterilised 30 ml Universal glass vials (Fisher Scientific, Cat. BTS-160-016G) containing 6 ml KB, which were incubated at room temperature (18–20°C) with shaking, using an automatic shaker system or statically (e.g. on a bench without disturbance). In both incubation conditions the lids were held loosely in place with tape in order to allow good aeration. After being shaken overnight, KB cultures were used to provide inocula for different experiments.

Sample dilution and plating

In order to simplify the dilution of bacterial samples to determine bacterial numbers, decimal dilutions were always employed. A 100 µl aliquot of the sample was added to 900 µl of KB liquid medium and mixed well to produce the '10^1' dilution. A 100 µl aliquot of the 10^1 dilution was added to a second 900 µl of KB to produce the '10^2' dilution, and this process continued down to the 10^8 dilution. In order to plate bacteria, 100 µl samples of the 10^6–10^8 dilutions were placed onto KB agar plates and spread across the surface using a glass or disposable-plastic spreading bar. Plates were incubated for two to three days before inspection. If five colonies were found on the 10^7 dilution plate, this would correspond to 5×10^8 colony forming units (cfu)/ml in the original sample (i.e. 10 (spreading)$\times10^7$ (dilution factor)), and 3×10^9 cfu per 6 ml microcosm.

Adaptive radiation

Replicate microcosms (n = 5) inoculated with 10 µl SBW25 culture were incubated statically or with shaking for five days. These were destructively sampled at different time points (e.g. after one, three and five days) by vigorous shaking by hand or using a bench-top vortex mixer (e.g. 30 s high-speed vortexing). Serial dilutions were made and the 10^6–10^8 dilutions plated out in order to determine total numbers and the percentage of WS in the population.

Niche colonisation

Microcosms inoculated with 100 µl of SBW25 or WS cultures (or colony samples obtained from KB agar plates using a wire loop) were incubated statically for

2–3 days. Bacterial growth in the liquid column and at the A–L interface was monitored visually and examination of microcosm contents poured into empty Petri dishes used to confirm the presence of biofilms.

Fitness assays

Relative competitive fitness (W) was determined by combining WS and ancestral SBW25 together in the same microcosm and monitoring the effect of competition between each culture. First, a mixture of 1:1 SBW25 and WS was made using 500 µl of the two overnight KB cultures. Aliquots of 10 µl of this mixture were used to inoculate replicate microcosms ($n = 3$) for the fitness assays. At the same time, 10^6–10^8 dilutions of the mixture were spread onto KB agar plates in order to determine the initial (i) numbers of WS and SBW25 cells added to the microcosms in the 100 µl aliquot of the mixed cultures. The fitness microcosms were incubated for three days before destructive sampling, dilution and plating to determine the final (f) numbers of each of WS and SBW25. W is determined by using the following equation: $W = ln [WS_f/WS_i]/ln [SBW25_f/SBW25_i]$.

Results

Wrinkly Spreaders evolve in static microcosms within days

The adaptive radiation of wild-type SBW25 and the evolution of the Wrinkly Spreaders (WS) is readily demonstrated in microcosms. Wild-type or 'ancestral' SBW25 is used to inoculate microcosms containing 6 ml King's B liquid medium which can then be incubated for three to seven days under static or shaking conditions. In Figure 3 we provide quantitative data from a five-day experiment. Over this period, the total number of bacteria increased from ~10^4 to 5×10^{10} in shaken microcosms, with significantly lower population levels seen in static microcosms where poor aeration has the effect of limiting growth (Figure 3A). During this population expansion, mutations accumulate. Evidence of this radiation can be seen in the diversity of colony morphologies where size, colour and texture can be used to distinguish different classes of mutants ('morphs') including the Wrinkly Spreaders. The percentage of Wrinkly Spreaders after five days is shown in Figure 3B and is significantly different between static and shaken microcosms (t-test, $P = 0.0014$). Static microcosms favour the development of WS mutants from the ancestral SBW25 population. In contrast, the shaken microcosm does not favour the development of WS mutants, and as a result they are rarely found under such conditions. WS mutants isolated from the adaptive radiation experiment can be used to determine niche preferences and fitness differences as described below.

Wrinkly Spreaders are novel colonisers of the A–L interface of static microcosms

Fresh KB microcosms can be inoculated with SBW25 and WS to determine niche preferences and to demonstrate WS colonisation of the A–L interface by

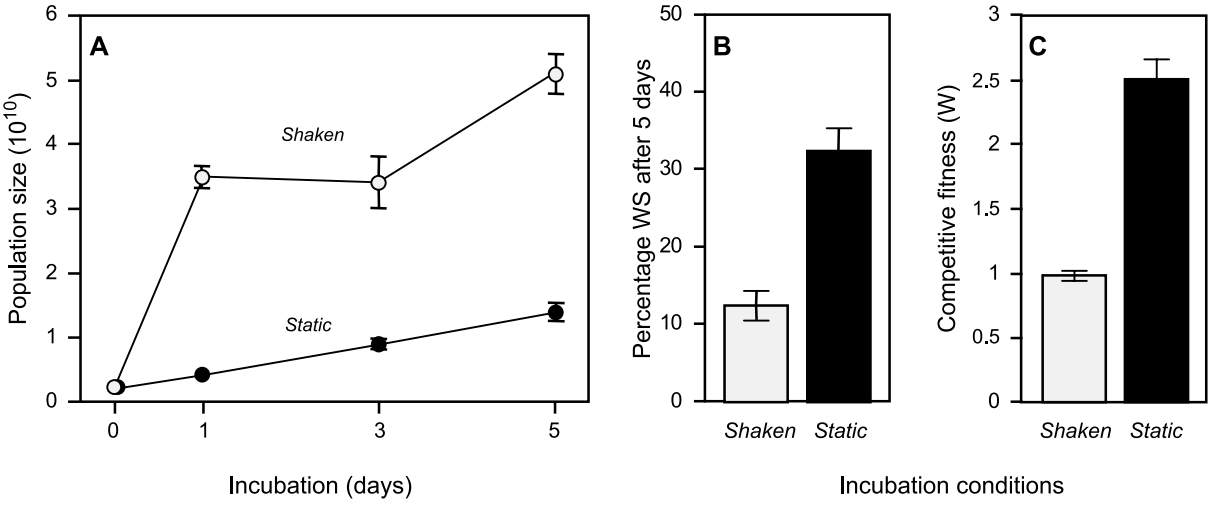

Figure 3. Evolution of the Wrinkly Spreader from populations of ancestral SBW25 in static and shaken liquid microcosms

Notes: Mutants such as the Wrinkly Spreader (WS) will accumulate in an expanding SBW25 population over time. (A) Population growth is faster in shaken liquid microcosms than in static microcosms due to better aeration (these microcosms were inoculated with ~10^4 ancestral SBW25 cells at the beginning of the experiment). (B) However, after 5 days, the percentage of WS is higher in static liquid microcosms than in shaken microcosms. (C) The evolved WS has a competitive fitness (W) advantage compared to the ancestral SBW25 in static liquid microcosms where biofilms can occur (i.e. W > 1). In contrast, in shaken liquid microcosms where there is no advantage to biofilm formation, the evolved WS and ancestral SBW25 have similar levels of fitness (W = 1). Means and SE are shown for each of A, B and C.

biofilm formation. The ancestral SBW25 shows a clear preference for the liquid column of static microcosms (Figure 1A). In contrast, the evolved WS will tend to grow poorly in the liquid column but produce a biofilm at the surface within two to three days (Figure 1B), demonstrating a clear difference in niche preference compared to SBW25.

Wrinkly Spreaders are fitter than the ancestral SBW25 in static microcosms

The ability of WS to colonise the A–L interface of static liquid microcosms provides a fitness advantage over the ancestral SBW25 which is unable to occupy this environment. Fitness (W) differences can be readily determined by competitive assay where both SBW25 and WS are cultured together. When $W > 1$, the WS has a fitness advantage over the ancestral SBW25, i.e. greater benefit (faster rate of growth) than cost (expense of producing cellulose for the biofilm); when $W = 1$, the two strains are equal; and when $W < 1$, the WS suffers a fitness disadvantage, i.e. greater cost than benefit. In static microcosms, $W = 2.51 \pm 0.15$ (mean ± standard error), suggesting that there is benefit with little cost to biofilm formation in this environment (Figure 3C). In contrast, in shaken microcosms, $W = 0.98 \pm 0.03$, suggesting that there is no benefit in trying to produce a biofilm in these conditions. The difference in W when comparing static with shaken microcosms is significant, as determined by t-test ($P = 0.0547$).

Discussion

The adaptive radiation of *Pseudomonas fluorescens* SBW25 in static King's B liquid medium microcosms is an ideal system with which to demonstrate evolution in secondary schools and tertiary-level practical classes. The evolution experiment illustrates the accumulation of mutants over time, and in turn provides WS mutants with which to demonstrate niche preference changes and fitness differences between the evolved WS and the ancestral SBW25. The adaptive radiation of SBW25 has parallels with topical medical and ecological concerns. Examples include bird and swine flus which jump the species barrier to infect people, hospital-acquired infections where pathogens move between patients in adjacent beds, the unwanted invasion by American crayfish of streams, and the deliberate reintroduction of sea eagles and choughs in the UK.

The experiments outlined in this paper require standard microbial equipment and the use of simple techniques. It should be noted that the evolution and fitness experiments in particular rely on accurate dilution and plating skills. However, we believe that these practical skills can be taught within the context of these experiments (our approach is to use more than the minimum number of replicates indicated to allow for errors). Both the evolution experiment and the fitness tests provide opportunity for statistical analyses of quantitative data, whilst photography of colony morphs, developing microcosms and biofilm material could also be added to produce detailed student or class reports. Furthermore, the strength of biofilms can be quantified using glass balls and the biofilm cellulose fibres can be visualised using fluorescent microscopy (see Spiers and Rainey 2005). It should also be noted that population growth and rates of WS accumulation are sensitive to incubation conditions and will differ between experiments, and that WS fitness will also vary depending on the individual WS mutants chosen for study (in particular, a $W < 1$ in shaken microcosms would not be unexpected). We have prepared additional teaching support material including more explicit protocols, an extensive list of references, plus a collection of images describing aspects of this work which can be obtained by contacting AS. Although the primary literature describing the molecular biology of the WS phenotype may be difficult to approach, we believe that this could be linked to the practical experiments to provide a real demonstration of the modern evolutionary synthesis.

Finally, in a similar manner to other researchers who are interested in developing science teaching resources, we can provide access to SBW25 and primary literature, help with interpretation, and the development of protocols and teaching extensions suitable for secondary schools and tertiary-level biology education. We have listed in Table 1 a number of ideas based on the WS system which could form the basis for further teacher-led or student investigations (please contact AS if more details are required).

Acknowledgements

Jennifer H. Green is a student at Arbroath High School and undertook the experimentation presented here with Anna Koza during a summer studentship

Table 1. Ideas for student investigations

Intermediate levels of disturbance	Mix static microcosms briefly each day, or alternate between static and shaken conditions
Agar plates instead of liquid microcosms	Evolve stable WS-like mutants on agar plates where the WS is less fit than the ancestral SBW25
Altering nutrient levels	Use minimal glucose media or diluted KB to reduce nutrient levels
O_2 availability	Use microcosms with sealed lids, or layers of mineral oil to restrict O_2 diffusion
Balance between O_2 and nutrients	Use short fat or long and thin microcosms in order to change the balance between O_2 diffusion and nutrient access

organised with the help of TechFestSetPoint (www.techfestsetpoint.org.uk) with Andrew Spiers. Margaret Ritchie is a science teacher at Arbroath High School and Honorary Research Fellow, Edinburgh Napier University. The University of Abertay Dundee is a charity registered in Scotland, No: SC016040.

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Appendix: Suppliers

- Fisher Scientific (www.fisher.co.uk), Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK.
- BD Company (www.bdeurope.com), The Danby Building, Edmund Halley Road, Oxford Science Park, Oxford OX4 4DQ, UK.