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Protistan predation interferes with bacterial long-term adaptation to substrate restriction by selecting for defence morphotypes

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Abstract

Bacteria that are introduced into aquatic habitats face a low substrate environment interspersed with rare productive 'hotspots', as well as high protistan grazing. Whereas the former condition should select for growth performance, the latter should favour traits that reduce predation mortality, such as the formation of large cell aggregates. However, protected morphotypes often convey a growth disadvantage, and bacteria thus face a trade-off between investing in growth or defence traits. We set up an evolutionary experiment with the freshwater isolate *Sphingobium* sp. strain Z007 that conditionally increases aggregate formation in supernatants from a predator–prey coculture. We hypothesized that low substrate levels would favour growth performance and reduce the aggregated subpopulation, but that the concomitant presence of a flagellate predator might conserve the defence trait. After 26 (1-week) growth cycles either with (P+) or without (P−) predators, bacteria had evolved into strikingly different phenotypes. Strains from P− had low numbers of aggregates and increased growth yield, both at the original rich growth conditions and on various single carbon sources. By contrast, isolates from the P+ treatment formed elevated proportions of defence morphotypes, but exhibited lower growth yield and metabolic versatility. Moreover, the evolved strains from both treatments had lost phenotypic plasticity of aggregate formation. In summary, the (transient) residence of bacteria at oligotrophic conditions may promote a facultative oligotrophic life style, which is advantageous for survival in aquatic habitats. However, the investment in defence against predation mortality may constrain microbial adaptation to the abiotic environment.

Introduction

Bacteria from various environments are continuously introduced into freshwaters, for example by terrestrial run-off (Ruiz-González *et al.*, 2015). These microbes face challenging growth conditions, as organic substrates such as sugars or amino acids are only available at nanomolar concentrations in most natural waters (Kirchman, 2003). While the allochthonous bacteria may transiently thrive in substrate-rich microniches, such as suspended organic aggregates (Grossart & Simon, 1993), they also have to sporadically explore the pelagic zone despite low substrate availability. Thus, they need to maintain metabolic features such as motility (Grossart *et al.*, 2001) or chemotactic sensing (Stocker *et al.*, 2008) even at suboptimal conditions. This state markedly differs from the 'starvation' type of metabolism; it has been referred to as the 'hungry' mode of bacterial growth at limiting supply of substrates or nutrients (Ferenci, 2001). Many successful freshwater bacteria with planktonic and surface-attached subpopulations are well adapted to growth at both, high and low concentrations of organic compounds (Kasalicky *et al.*, 2013). It is conceivable that this metabolic versatility might be related to interspersed periods of 'hungry' growth at low substrate concentrations.

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Besides nutrients, the survival of microbes in aquatic habitats is strongly affected by protistan predation, in particular by bacterivorous nanoflagellates (Pernthaler, 2005). These predators can control total bacterial abundances and activity (Del Giorgio *et al.*, 1996), and their foraging is crucial for nutrient recycling (Caron *et al.*, 1988). Bacteria have responded to flagellate predation by evolving various defence strategies (Hahn & Höfle, 2001; Jousset, 2012). Morphological adaptations include the development of a permanently small or large cell size (Pernthaler *et al.*, 2004; Salcher *et al.*, 2011), and the formation of large, inedible cell aggregates or microcolonies may protect a subpopulation from flagellate grazing (Hahn *et al.*, 2004; Blom *et al.*, 2010a).

Such adaptations might come with a fitness cost. For example, densely packed cells within flocks or biofilms might experience reduced diffusive flux, leading to a growth disadvantage reminiscent of intraspecific 'shadow' competition (Lubin *et al.*, 2001). Biofilm-associated *Streptococcus mutants* have been shown to grow more slowly than planktonic cells (Welch *et al.*, 2012). To avoid these costs, bacteria may resort to phenotypic plasticity for the expression of defence morphotypes: both, the formation of filaments and aggregated cells can be conditionally induced by a signal from the predator (Corno & Jürgens, 2006; Blom *et al.*, 2010b). Theoretical analyses suggest that long-term evolutionary pressure imposed by intraspecific substrate competition should either lead to the loss or the constitutive fixation of such physiological trait variability if predators are permanently absent or present, respectively (Yamamichi *et al.*, 2011).

Sphingobium spp. and related genera are consistently found both in the pelagic zone of freshwaters and attached to organic particles (Bizic-Ionescu *et al.*, 2015). Members of this genus are also prevalent in treated drinking water and are suspected to be a reservoir of antibiotic resistance (Vaz-Moreira *et al.*, 2011). The freshwater bacterial isolate *Sphingobium* sp. Z007 increases the formation of aggregates in the presence of a bacterivorous nanoflagellate (Blom *et al.*, 2010a). Supernatants of a predator–prey coculture also induce this behaviour, indicating the involvement of an infochemical (Blom *et al.*, 2010b). While the strain has been isolated from central Lake Zurich waters, its closest relatives are from habitats such as soil or the rhizosphere (Pal *et al.*, 2005).

We set up an experiment with *Sphingobium* sp. Z007 and the bacterivorous nanoflagellate *Poterioochromonas* sp. strain DS to test the hypothesis that aggregate formation would be disadvantageous at low substrate concentrations and would be rapidly lost in the absence of the inducing stimulus. Moreover, we hypothesized that the advantage of avoiding predation mortality might outweigh the growth penalty of the aggregated state, and the presence of predators would therefore rescue this behaviour. Bacteria were grown at oligotrophic conditions in semicontinuous culture (weekly re-inoculations) with and without predators for 29 (1-week) growth cycles.

Materials and methods

Microbial strains and cultivation conditions

Sphingobium sp. strain Z007 (EMBL 16S rRNA gene accession number, FN293045) was originally isolated from the surface water of mesotrophic Lake Zürich in 2006 (Blom & Pernthaler, 2010). It is a Gram-negative, rod-shaped bacterium with the ability to form large aggregates/microcolonies. Stock cultures of this strain were preserved at −80 °C in 50% v/v glycerol (Sigma, Buchs, Switzerland). For the experiments, it was first regrown in nutrient-rich DSMZ 7 medium (yeast extract 1 g L^{-1}; glucose 1 g L^{-1}; peptone 1 g L^{-1}; German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany) in 300-mL Erlenmeyer flasks at 18 °C in the dark. Bacteria were transferred into fresh medium 72 h before starting the experiments. During the evolution experiment, strains were isolated at different time points. These isolates were also preserved at −80 °C in 50% glycerol and regrown in DSMZ 7 medium as described above for further experiments. Axenic cultures of the bacterivorous mixotrophic flagellate predator *Poterioochromonas* strain DS were used for the experiments (Blom *et al.*, 2010b). Stock cultures of the flagellates were maintained in nutrient-rich '*Ochromonas* medium' (yeast extract 1 g L^{-1}; meat extract 1 g L^{-1}; glucose 1 g L^{-1}; peptone 1 g L^{-1}; Culture Collection of Algae at the University of Göttingen, Germany) at 18 °C in the dark, and flagellates were fed weekly with heat killed bacteria from a different species (*Flectobacillus major* DSMZ 103, 1×10^7 cells mL^{-1}; pre-incubated at 70 °C for 2 h). Prior to the experiments, flagellate cultures were inoculated into fresh medium and starved for 72 h.

The evolution experiment was carried out in nutrient-poor oligotrophic medium. Artificial lake water (ALW), a mix of inorganic nutrients and trace elements (Zotina *et al.*, 2003), was amended with the substrates of the DSMZ 7 medium at 1 : 1000 dilution (yeast extract 1 mg L^{-1}; glucose 1 mg L^{-1}; peptone 1 mg L^{-1}). All other experiments were performed in DSMZ 7 medium. For isolation of strains on plates, DSMZ 7 medium was amended with 16.4 g L^{-1} of agar.

Quantification of free bacteria, aggregated cells and flagellates

Samples for flow cytometry were stained with DAPI (4,6-Diamidino-2-phenylindole, Serva, Heidelberg, Germany, 1 μg mL^{-1}) for 30 min in the dark and analysed

using an InFlux V-GS cell sorter (Becton Dickinson Inc., San Jose, CA, USA). A UV laser (60 mW, 355 nm; CY-PS; Lightwave Electronics, Mountain View, CA, USA) was used for detection of DAPI fluorescence, and a blue laser (200 mW, 488 nm; Sapphire; Coherent Inc., Santa Clara, CA, USA for scattered light and autofluorescence of flagellates. If required, samples were diluted with deionized water prefiltered with a 0.2-μm pore size filter to avoid particle coincidence. Data analysis was carried out with an in-house custom software (J. Villiger & J. Pernthaler, unpublished). Single cells of *Sphingobium* sp. strain Z007 were identified using side-scattered light (SSC) vs. DAPI fluorescence (431 nm). *Poterioochromonas* sp. strain DS was identified using SSC vs. green fluorescence (531 nm). Flagellates were separately gated and excluded from the counts of aggregates. Aggregates were operationally defined by fluorescence and scatter properties equal to or higher than that of *Poterioochromonas* sp. strain DS (Blom *et al.*, 2010b). This definition and the proper detection of flagellate cells were verified by flow cytometric sorting of events in the respective gates in the cytograms and subsequent microscopic inspection.

Evolution experiment

The evolution experiment was carried out with bacterial cultures (treatment P−) and cocultures of bacteria and flagellates (treatment P+) by serial propagation under semicontinuous conditions in ALW medium. The experiment was run for 200 days (approximately 29 weeks) in 300-mL Erlenmeyer flasks in a volume of 100 mL medium at 18 °C in the dark. Five sequential batch cultures were inoculated only with *Sphingobium* sp. strain Z007 (initial densities 1.5×10^6 cells mL^{-1}), or with *Sphingobium* sp. strain Z007 (initial densities 1.5×10^6 cells mL^{-1}) and *Poterioochromonas* sp. strain DS (initial densities, 1.5×10^3 cells mL^{-1}). All cultures were re-inoculated weekly. For this purpose, 10 mL of the cultures or cocultures was added to 90 mL of fresh ALW medium. Subsamples of 1 mL were taken at three time points (72, 120 and 168 h) and fixed with glutaraldehyde (final concentration, 2.5%, Sigma) to determine the abundances of bacterial single cells, aggregates and flagellates. Samples were stored at 1 °C until they were analysed by flow cytometry (usually within 24–48 h).

Starting from day 18, subsamples were taken biweekly from each of the ten Erlenmeyer flasks and plated on DSMZ 7 medium agar plates, to separate bacteria from flagellates (in the coculture treatments). The conspicuous yellow colour of the *Sphingobium* sp. colonies helped to confirm the absence of contaminants. After 3 days, three bacterial colonies per flask were picked from the plates, suspended in 1 mL glycerol (50%; Sigma) and stored at −80 °C until further analyses. These isolates are denominated according to treatment (P+ or P−) and the number of growth cycles (weeks) they spent in the evolution experiment (e.g. 7, 16, 26 cycles).

Measuring evolution of intrinsic aggregation and biofilm formation

The numbers of free cells and aggregates after 72 h of growth (as described below in section 'Measuring evolution of growth performance at rich conditions') were determined for the ancestor and for five evolved strains isolated at 10 time points from different vessels of each treatment of the evolution experiment. To determine the ability of evolved *Sphingobium* strains to form biofilms, isolates from both treatments from growth cycle 7, 16, and 26 were grown in DSMZ 7 medium for 5 days at room temperature. Bacteria were re-inoculated into DSMZ 7 medium to a final concentration of approximately 2×10^5 mL^{-1}, transferred into a 96-well plate and incubated in the dark at 18 °C. All isolates were measured in triplicate. After 72 h of growth, all wells were incubated for 10 min with 40 μL of a 0.1% w/v crystal violet solution (Merck, Darmstadt, Germany). Subsequently, the liquid phase was discarded and the wells were washed three times with PBS buffer (50 mM; pH 7.4). After drying for 15 min at room temperature, 200 μL of 95% ethanol was added to each well to solubilize the precipitated crystal violet. The solvent was mixed well by pipetting and incubated for 15 min, and absorption was measured at 600 nm with an absorption microplate reader (Spectra Max 190; Molecular Device Corporation, Sunnyvale, CA, USA).

Measuring evolution of bacterial defence

Sphingobium strains were cocultivated with *Poterioochromonas* sp. strain DS following the set-up described by Blom *et al.* (Blom *et al.*, 2010a). Experiments were carried out with the ancestor strain and with strains obtained from three time points (7, 16 and 26 growth cycles) of both treatments of the evolution experiment. Unless otherwise stated, isolates from the same three vessels of each treatment were used in all experiments described below. Individual sets of batch culture experiments were performed with six strains that each originated from a different vessel of the evolution experiment (3 from P+ and 3 from P−) and with three separately picked colonies of the ancestor strain. Bacteria and flagellates were inoculated at initial densities of 1.0×10^6 mL^{-1} and 1.0×10^3 mL^{-1}, respectively. Control experiments were performed without addition of predators. All experiments were carried out in 300 mL Erlenmeyer flasks in a final volume of 50 mL DSMZ 7 medium at 18 °C in the dark. Subsamples of 1 mL were taken every 24 h until the end of the experiment (72 h) and fixed with glutaraldehyde (final

concentration 2.5%). Samples were stored at 1 °C until further analysis by flow cytometry (within 24–48 h).

Measuring plastic response to predation of evolved strains

Bacterial strains isolated after 26 growth cycles from both treatments of the evolution experiment were tested for their ability of induced aggregate formation by a chemical cue. The set-up for these experiments basically followed the one described in Blom *et al.* (Blom *et al.*, 2010b). Briefly, the *Sphingobium* sp. Z007 ancestor strain was cocultivated with *Poterioochromonas* sp. strain DS for 120 h (initial densities of bacteria and flagellates, 1.0×10^6 mL^{-1} and 1.0×10^3 mL^{-1}) in DSMZ 7 medium. Subsequently, cultures were centrifuged two times for 15 min at 9000 *g* under sterile conditions. The pellet was subsequently discarded and the supernatants were frozen at −20 °C for at least 1 h to destroy any remaining flagellate cells. Immediately after thawing, the supernatants were filled in 24-well plates and inoculated with 1.0×10^6 mL^{-1} of the evolved strains (final volume, 2.0 mL). Control treatments were supernatants of *Sphingobium* sp. Z007 cultures without addition of flagellates. Subsamples (500 μL) were taken after 48 h, fixed with glutaraldehyde (final concentration 2.5%) and analysed by flow cytometry.

Visualization of aggregate structure and matrix

For the staining of cell aggregates with fluorescently labelled lectins (Neu *et al.*, 2001), a bacterial strain was used that had been isolated after 16 growth cycles from treatment P+. Bacteria from the −80 °C stock cultures were grown for 120 h in DSMZ 7 medium. When appropriate, the cells in the aggregates were counterstained with the nucleic acid-specific dyes SYTO 9 or SYTO 60 and Sypro Red or FM 4-64 (Molecular Probes, Eugene, OR, USA). To identify a suitable panel of lectins for glycoconjugate staining, 77 fluorescent lectins were tested. The lectins (Sigma, Buchs, Switzerland, and Vector Laboratories, EY Laboratories, San Mateo, CA, USA) were labelled either with the fluorescein isothiocyanate (FITC), tetramethyl rhodamine isothiocyanate (TRITC), Alexa-488 or Alexa-568. Lectins were dissolved in a solution of 0.1 mg mL^{-1} in filter-sterilized (0.2 μm) H_2O. The samples (500 μL of bacterial culture) were mixed with 500 μL of a solution with fluorescent lectins and stained for 20 min in the dark at room temperature. Subsequently, the bacterial aggregates were carefully washed three times with deionized H_2O. After the last centrifugation step, the bacterial aggregates were suspended in deionized H_2O, transferred to a glass microscope slide and covered with a coverslip for optimal fluidity of the samples. The sample drop volume was adjusted to 20 μL to minimize the floating of bacterial aggregates. Samples were examined under a confocal laser scanning microscope (Leica TCS SP5X, Wetzlar, Germany) equipped with an upright microscope and a super continuum laser source (470–670 nm). The system was controlled by the LAS AF version 2.6.1. software (Leica, Wetzlar, Germany) Excitation lines were selected according to the excitation maximum of the fluorochromes. Emission signals were recorded at the point (±5 nm) of excitation (reflection) or with a distance of 10–15 nm from excitation towards the longer wave length. Images were deconvolved using Huygens version 15.10.1p2 (SVI, Hilversum, the Netherlands) and projected with Imaris version 8.2.0 (Bitplane, Zurich, Switzerland).

Measuring evolution of growth performance at rich conditions

We tested whether the maintenance at oligotrophic conditions had changed the growth performance of the evolved strains on the original, rich medium. Growth curves were determined of the ancestor strain and of evolved isolates obtained from 10 time points of the evolution experiment. Three separate colonies of the ancestor strain were analysed, as well as three evolved strains per time point and treatment, each obtained from a different experimental vessel. Colonies were suspended in DSMZ 7 medium to a final concentration of approximately 1×10^5 mL^{-1}. Bacterial growth was measured as optical density at 600 nm (OD_{600}) in a absorption plate reader (Spectra Max 190; Molecular Device Corporation) every 60 min for 72 h. The growth curve of every strain was measured in triplicate. Mean values and standard errors of all replicate measurements were calculated for each time point. OD measurements from the ancestor and the evolved strains were considered comparable because the size of free cells did not visibly differ between treatments (as assessed from their respective flow cytometric signatures, data not shown). The duration of the lag phase preceding exponential growth was determined by first normalizing the individual growth curves and then fitting them with the Gompertz model (Zwietering *et al.*, 1990) with the help of the online tool 'interactive platform for analysis of microbial growth' (https://statu.shinyapps.io/ExploreMicrobialGrowth/).

Measuring evolution of metabolic versatility and of growth on single substrates

The *Sphingobium* sp. strain Z007 ancestor and six strains isolated after 26 growth cycles from different vessels of the evolution experiment (3 from P+ and 3 from P−) were streaked on DSMZ 7 agar plates and incubated at 18 °C in the dark. Colonies were carefully picked after 72 h, avoiding nutrient contamination from the agar plates, suspended in 2 mL of ALW, and OD_{600} was

adjusted to 0.18 (Cary 3, Varian). About 1.5 mL of the suspension was diluted with 13.5 mL of ALW. About 100 μL of the diluted suspension was transferred to each well of the PM1 and PM2 substrate array plates (Biolog, Hayward, CA, USA). The plates were incubated in the dark at 18 °C. After 48 h of incubation, 1.5 μL of the redox dye A (Biolog) was added, and plates were incubated for another 24 h at the conditions described above. Individual wells were then tested for respiratory activity by measuring optical density at 590 nm (OD_{590}) – the absorption maximum of the redox dye – with an absorption microplate reader (Spectra Max 190; Molecular Devices, Sunnyvale, CA, USA). The integrated OD_{590} value of the negative control wells was then subtracted from each measurement. Only background-corrected OD_{590} values > 0.05 were considered as positive growth.

After establishing the substrate spectra qualitatively, we also quantitatively compared growth of the ancestor and evolved strains on a subset of single substrates. For this purpose, the above assays were carried out without the potentially toxic redox dye (Ullrich *et al.*, 1996) on substrates utilized by all strains. Plates were incubated for 72 h at 18 °C in an absorption microplate reader (Spectra Max 190; Molecular Devices) that recorded OD_{600} every 15 min. Prior to every read the plate was shaken for 3 s. OD_{600} readings were corrected by the corresponding values of negative controls (bacteria incubated in substrate-free ALW). The area under the growth curve (AUC) was determined as a proxy of total growth yield. The duration of the lag phase was determined as described above.

Statistical analyses

Differences between the maximal OD_{600} reached by the ancestor and the evolved strains on complex medium (pooled data of isolates from three time points) were analysed by two-sided Dunnett's tests of 10 measurement points per curve during the early stationary phase before OD started to decrease due to death of cells (after 30 h of growth for the ancestor and 50 h of growth for the evolved strains). The same test was used to examine the differences between the length of lag phases and between the numbers of single cells, aggregates or the ratios of aggregates to free cells. Differences between P+ and P− strains were examined by Tukey's tests (numbers of single cells and aggregates at individual isolation time points) or by either unpaired (growth curve parameters) or paired (numbers of single cells or aggregates at all isolation time points) *t*-tests. For the comparison of growth yield on different substrates and the duration of the lag phase, the results from individual substrates were pooled into the classes amino acids ($n = 9$), carbolic acids ($n = 5$), dipeptides ($n = 3$), monosaccharides ($n = 6$) and oligosaccharides ($n = 4$). First, a two-way ANOVA with the dependent variables strain type and substrate class was performed. After establishing that the two factors as well as their interaction were significant at $P < 0.05$, the individual differences between the ancestor and the evolved strains were assessed by Dunnett's tests. In the supernatant experiment, we tested for differences between the treatments by Tukey's range tests. Statistical analyses were performed on untransformed data if no significant deviation from normalcy was detected (Kolmogorov–Smirnov tests) and on log-transformed data otherwise. The ratios of aggregates to free cells were normalized by a logit-transformation. All statistical analysis was carried out with SPSS (IBM Analytics, Armonk, NY, USA).

Results

Population dynamics during evolution experiment

The endpoints of the growth curves of each growth cycle (1 week or 168 h) are depicted in Fig. 1. In the predator-free environment (P−), the final numbers of free bacterial cells increased from 3×10^6 mL^{-1} to approximately 20×10^6 mL^{-1} after 10 weeks (Fig. 1a) and remained stable at $19 \pm 3.7 \times 10^6$ mL^{-1} thereafter. With the exception of one peak after 10 growth cycles (1.8×10^3 mL^{-1}), the numbers of aggregates did not change significantly between the beginning and the end time points in this treatment (mean, $0.26 \pm 0.07 \times 10^3$ mL^{-1}, Fig. 1b). In cocultures of *Sphingobium* sp. strain Z007 and *Poterioochromonas* sp. strain DS (P+), the numbers of flagellates stabilized at $5.6 \pm 1.6 \times 10^3$ mL^{-1} already after 2–3 growth cycles; while there was no significant increase of flagellate numbers thereafter, we observed one conspicuous peak towards the end of the experiment (Fig. 1a). The abundances of free bacterial cells in P+ initially dropped to 0.05×10^6 mL^{-1}, but increased gradually over the course of the experiment, to eventually 1.4×10^6 mL^{-1}; their average numbers were significantly higher during the last five growth cycles than during the first five-ones (unpaired *t*-test, $t_8 = -9.03$, $P = 0.001$).

The number of aggregates in the bacteria–predator coculture was always significantly higher in P+ (2.03×10^3 mL^{-1}) than in P− (0.31×10^3 mL^{-1}) (unpaired *t*-test, $t_{54} = -11.48$, $P = 4.52 \times 10^{-14}$). It transiently increased in the period between 8 and 14 growth cycles and again from week 22 until the end of the experiment ($3.1 \pm 0.69 \times 10^3$ mL^{-1}, Fig. 1b). These fluctuations, however, were not statistically significant due to the relatively large variations between replicates.

Microscopic analysis of the defence phenotype

Twenty-nine lectins were found to bind to aggregate cells and matrix material, of which 13 had a strong

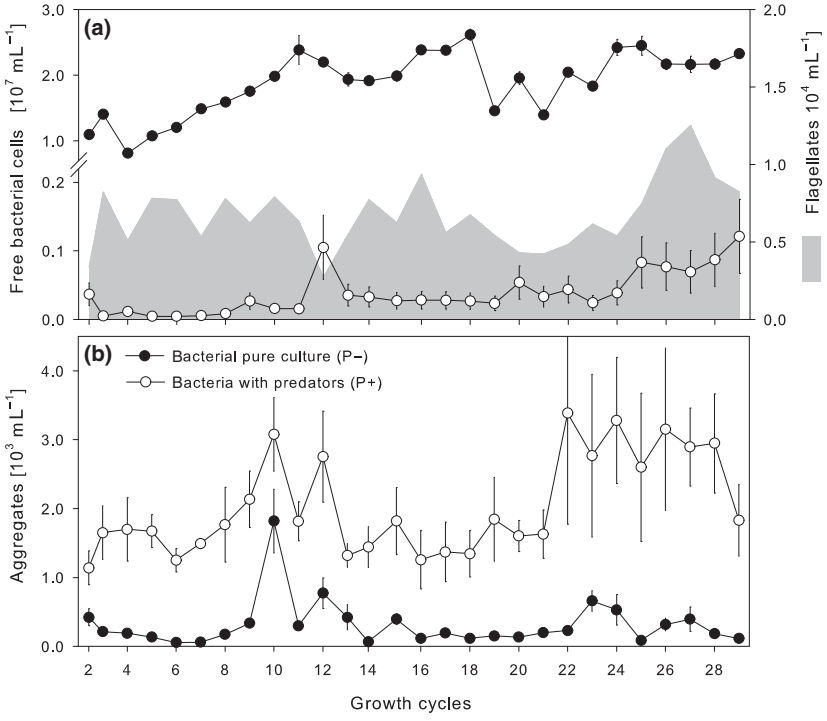

Fig. 1 Numbers of (a) free cells and (b) aggregates of *Sphingobium* sp. strain Z007 after 168 h of growth during sequential batch cultivation in 1 : 1000 diluted substrate concentration either in pure culture (P−) or together with the nanoflagellate *Poterioochromonas* sp. strain DS (P+). The numbers of flagellates are depicted as grey area in the upper panel.

and 16 had a weaker signal (Table S1). The panel of positive lectins revealed a differential binding pattern to the *Sphingobium* aggregates. Examination by confocal laser scanning microscopy showed the overall structure of the aggregates in reflection mode (Fig. 2a). Cellular distribution as well as cell dimensions and lengths were demonstrated by protein (Fig. 2b) and membrane staining (Fig. 2c). The lectin glycoconjugate signals were located at the cell surface (Fig. 2d) or throughout the aggregate indicating a glycoconjugate matrix (Fig. 2e). Combinations of reflection imaging, nucleic acid and glycoconjugate staining qualitatively showed the clustered composition of the larger aggregates (Fig. 2f). Using the lectins AAL, HPA and VVA in combination with nucleic acid staining and reflection imaging, the polar excretion of glycoconjugates could be demonstrated. These sticky glycoconjugates are crucial for building star-like cell aggregations with few bacteria as an initial stage of larger mature clusters (Fig. 2g–i).

Evolution of the aggregated subpopulation and of phenotypic plasticity

Sphingobium sp. strains that were isolated after seven growth cycles from either treatment of the long-term evolution experiment showed increased biofilm formation compared to the ancestor strain (Fig. 3a). By contrast, isolates from the last time point (26 growth cycles) clearly differed in their response according to their respective origin: there was significantly lower crystal violet precipitation in samples of isolates from the P− treatment (Fig. 3a, Dunnett's test, $I\text{-}J_{14} = -1.76$, $P = 0.00057$), whereas biofilm formation remained higher than that of the ancestor in the corresponding strains from the P+ treatment (Fig. 3a, Dunnett's test, $I\text{-}J_{14} = 1.97$, $P = 0.00018$).

Isolates from the P− treatments formed up to four times more free cells than those from the P+ treatments (Fig. 3b). These differences were already highly significant after seven growth cycles (Fig. 3b, Tukey's test, $I\text{-}J_{17} = 2.45 \times 10^{8}$, $P = 1.72 \times 10^{-11}$). In turn, P+ isolates always tended to form aggregates (Fig. 3c). Enhanced aggregate formation compared to the ancestor became highly significant in the P+ strains isolated after 12 growth cycles and later (Fig. 3c). There was a highly significant difference in aggregate formation between P+ and P− strains from all time points (paired *t*-test, $t_8 = 7.52$, $P = 0.00007$), and after 26 growth cycles, the number of aggregates in P+ strains was > 50 times higher than in the corresponding P− strains.

Phenotypic plasticity of aggregate formation was lost in the evolved strains: P+ strains had significantly higher ratios of aggregates to single cells (r_A) than the ancestor already after seven growth cycles when grown in an identical experimental set-up either without (Fig. 4a, Dunnett's test, $I\text{-}J_{13} = 0.0.942$, $P = 0.00002$) or with flagellates (Fig. 4b, Dunnett's test, $I\text{-}J_{13} = 1.285$, $P = 0.0013$). The opposite was the case for all P-strains

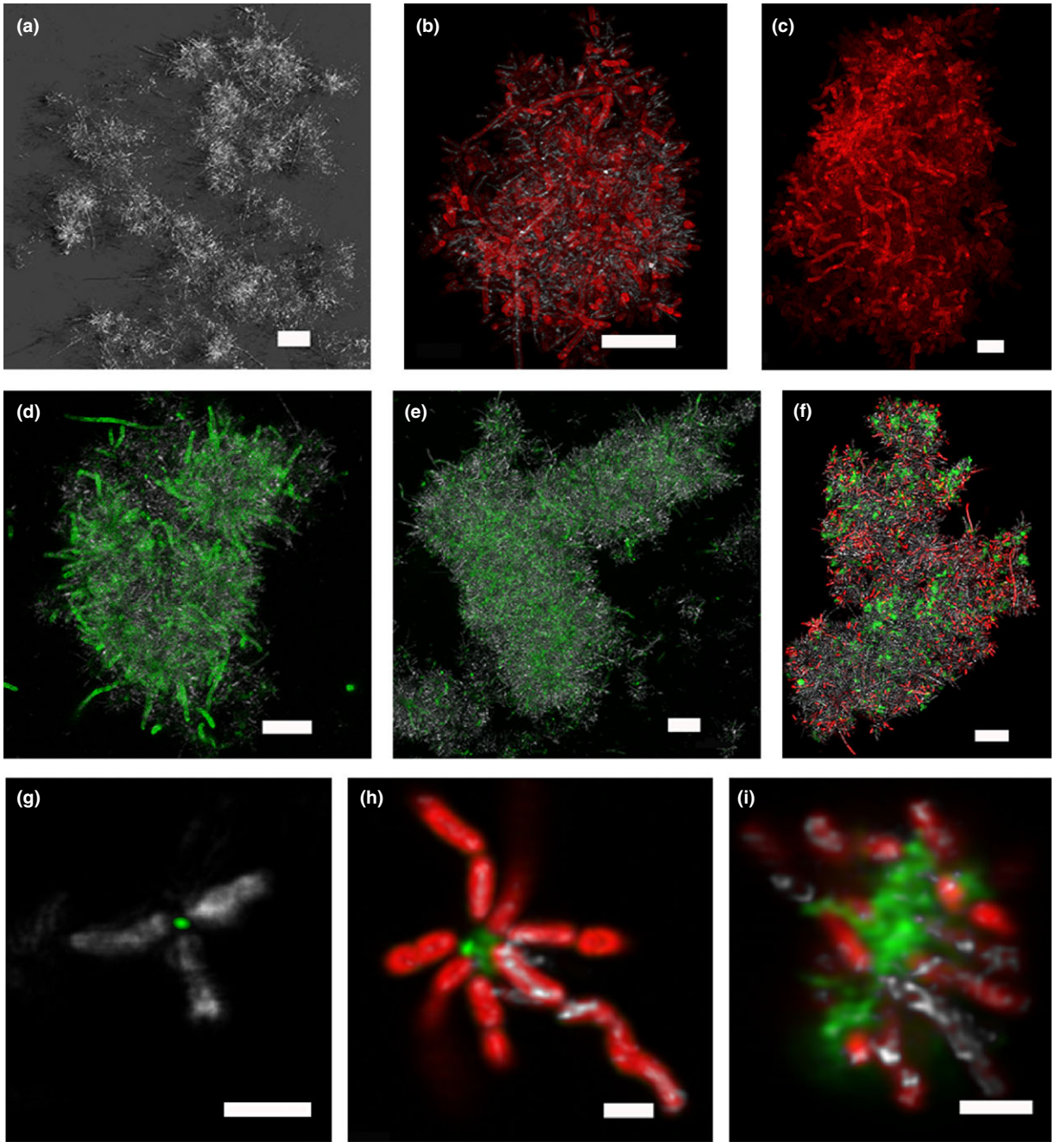

Fig. 2 (a–f) Maximum intensity projection of CLSM image data sets after deconvolution. (a) reflection image of star-like aggregates, (b) reflection and protein staining, (c) reflection and membrane staining, (d) reflection and GNA lectin bound to bacterial cell surface, (e) reflection and UDA lectin bound to aggregate matrix and (f) reflection with AAL lectin and nucleic acid staining. (g–i) Single scans after deconvolution showing early stages of bacterial star-like aggregates. The lectins identified the adhesive glycoconjugates in the centre of the stars. (g) VVA lectin, (h) AAL lectin, (i) HPA lectin. Colour allocation: grey – reflection; green – lectins; red – proteins (b), cell membranes (c) or nucleic acids (f, h, i). Scale bars: panels a–f, 10 μm; panels g–i, 2 μm.

(if grown without predators, Fig. 4a) or for those P– strains that had been isolated after 16 and 26 growth cycles (if grown with predators, Fig. 4b). Moreover, only the ancestor strain responded to supernatants from a predator–prey coculture with a significant increase of r_A, as compared to supernatants from a bacterial culture only (Fig. 4c, Dunnett's test, $I\text{-}J_{12} = -0.0.315$, $P = 0.046$).

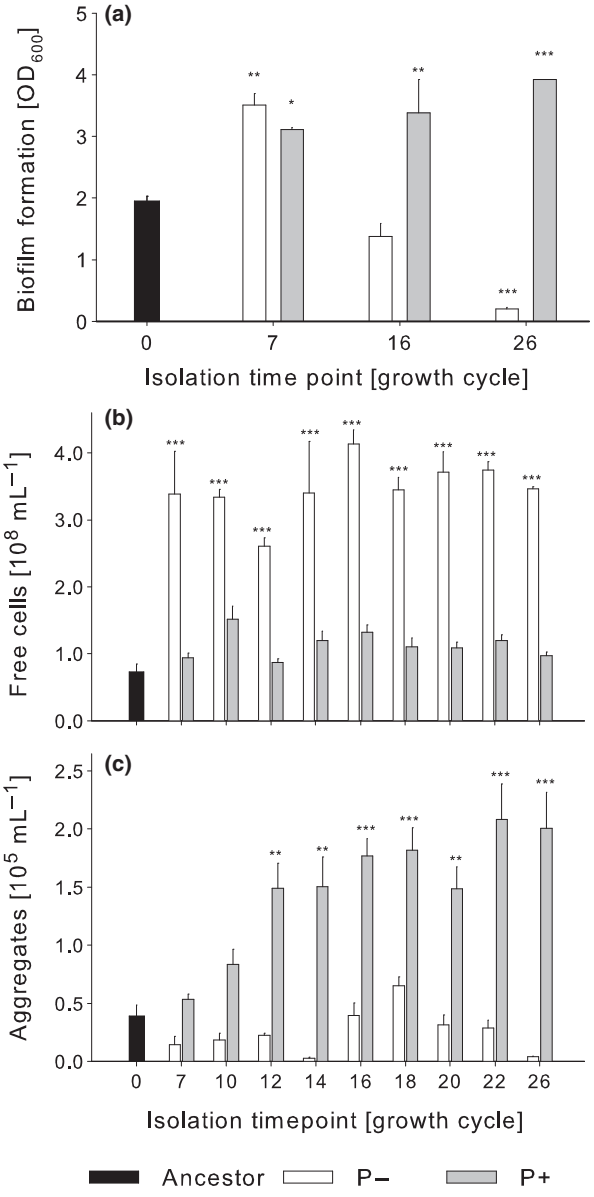

Fig. 3 (a) Biofilm formation of the ancestor after 72 h of growth in rich medium and of strains isolated at different time points from the evolution experiment, as determined by crystal violet staining. (b) Free cells and (c) aggregates of evolved bacterial strains analysed after 72 h of growth in rich medium. The strains were isolated at different time points (growth cycles) from three replicate vessels of each treatment of the evolution experiment. Error bars are 1 standard deviation ($n = 9$). $^{**}P < 0.01$; $^{***}P < 0.001$.

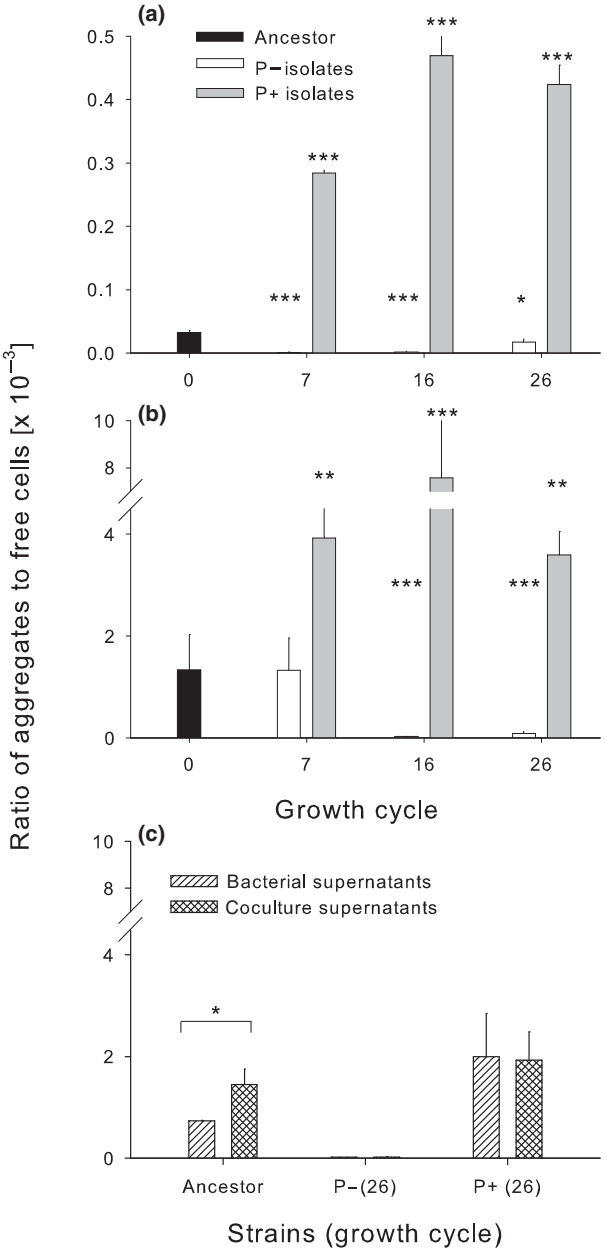

Fig. 4 Ratio of aggregates to free cells in the ancestor and in evolved strains isolated at different time points from treatments with (P+) and without (P–) predators. (a) Strains were grown in pure culture. (b) Strains were grown in direct contact with *Poterioochromonas* sp. strain DS. (c) Strains were grown in supernatants of either a pure culture of the ancestor strain or a coculture of the ancestor and the flagellates.

General growth costs of predation defence

After a lag phase of 13 ± 2 h, the *Sphingobium* sp. ancestor strain showed steep exponential growth. It reached stationary phase after about 27 h at an OD_{600} value of 0.52 (Fig. 5). Thereafter, OD_{600} slowly declined to < 0.4. For the sake of clarity, only the growth curves of strains isolated from three time points of the evolution experiment are depicted in Fig. 5. All evolved strains had a significantly longer lag phase on rich

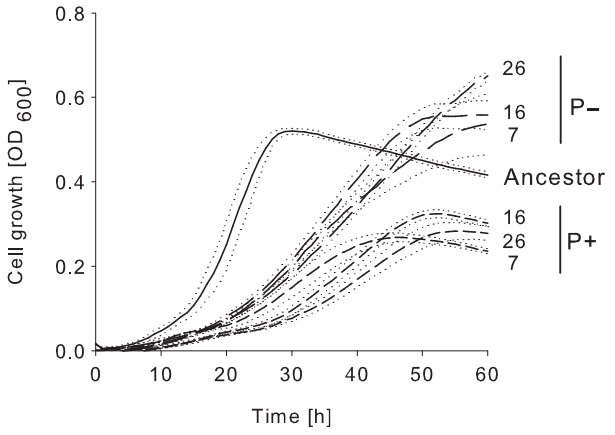

Fig. 5 Growth curves of the ancestor strain *Sphingobium* sp. strain Z007 on rich medium and of strains that evolved either without (P−) or with predation (P+) for 7, 16 and 26 growth cycles. The dotted lines represent 1 standard deviation of either three biological replicates (ancestor) or of three strains isolated from different replicate vessels per treatment (evolved strains).

media than the ancestor (Dunnett's tests, P− strains: $I\text{-}J_{26} = 7.28$, $P = 0.00005$; P+ strains: $I\text{-}J_{26} = 13.64$, $P = 1.5 \times 10^{-8}$), but they also had clearly contrasting growth patterns according to their treatment of origin, with little differences between isolates from different time points. The lag phase of the P− strains (21 h ± 2) was significantly shorter than that of the P+ strains (27 h ± 3) ($t_{37} = -8.5$, $P = 2.2 \times 10^{-10}$). Exponential growth of P− strains was slower than the ancestor, with strains isolated after 26 growth cycles not even reaching the stationary phase within the 70 h of the experiment. At the same time, their maximal OD_{600} was significantly higher than that of the ancestor strain (Dunnett's test, $I\text{-}J_{74} = 0.12$, $P = 1.84 \times 10^{-8}$). The isolated strains from the P+ treatments grew most slowly, and reached significantly lower maximal OD_{600} values (0.32) than the ancestor (Dunnett's test, $I\text{-}J_{74} = -0.15$, $P = 1.84 \times 10^{-8}$).

Costs of defence for metabolic versatility and growth on single substrates

The ancestral *Sphingobium* sp. strain Z007 showed unambiguous strong growth (i.e. in all three technical replicates) on 28 substrates of the PM1 and PM2 microarrays, and growth on another 14 substrates was observed in one or (mostly) two replicates (Table S2). Highest OD_{590} was observed on glutamine, glutamate, citric acid and methyl pyruvate and on various mono- and oligosaccharides. In most instances, all three strains isolated from different replicates of the evolution experiment showed a similar positive or negative response to the offered substrates, but there were several instances (four in P+ and three in P−) when one of the strains diverged. More frequently, there were differences between the two technical replicates of individual isolates, likely due to a weak growth response at the lower detection limit of the method. In contrast to the ancestor, none of the evolved strains visibly grew on lactic acid. In addition, all isolates from the P+ treatments apparently lost the ability to grow on 4-hydroxy benzoic acid and D-mannose. By contrast, the P− strains showed growth on substrates where no growth of the ancestor could be detected (e.g. on the exotic monosaccharides D-fucose and L-lyxose).

All tested strains showed respiratory activity on a subset of 30 substrates. For 27 of these substrates, positive growth could also be unequivocally established without the addition of the redox dye, that is by direct measurements of OD_{600}. These substrates were therefore included in the subsequent quantitative comparison of growth parameters (Fig. 6). Strains from the P− treatment showed significantly higher growth yield than the ancestor in all but one substrate class (carbolic acids) (Fig. 6a). Incidentally, this was also the only category where P+ strains showed less growth than the ancestor. A significant reduction of the duration of the lag phase was also apparent in P− strains for some of the substrate classes (Fig. 6b).

Discussion

Adaptation to oligotrophy affects growth performance in rich conditions

Sphingobium sp. Z007 strains evolved at oligotrophic growth conditions (without predators) had drastically increased proportions of free single cells (Figs 1 and 3b). This phenotypic shift was not due to physiological acclimation; it was manifested by isolates resuscitated from frozen stocks and irrespective of predation (Fig. 4a,b). Concomitantly, the P− strains had increased growth yield at oligotrophic conditions (Fig. 1a, cycles 21–26 vs. cycles 1–5) and on a variety of single carbon sources (Fig. 6a). The passage through a bottleneck of oligotrophy also increased the ability of these strains to form biomass at high substrate concentrations (Fig. 5). This indicates that there was indeed a physiological cost to the lost defence trait (Fig. 3c).

The pelagic zone of freshwaters harbours numerous microscopic flocks of organic material that are densely colonized by bacteria (Grossart & Simon, 1993; Grossart *et al.*, 2003). These 'lake snow' particles are, for example, generated by the aggregation of senescent algal cells during the decline of phytoplankton blooms, and their concentration may greatly vary between seasons. Planktonic bacteria with free-living and attached growth forms are thus challenged to optimize their carbon utilization efficiency across a wide range of substrate concentrations. Our findings suggest that

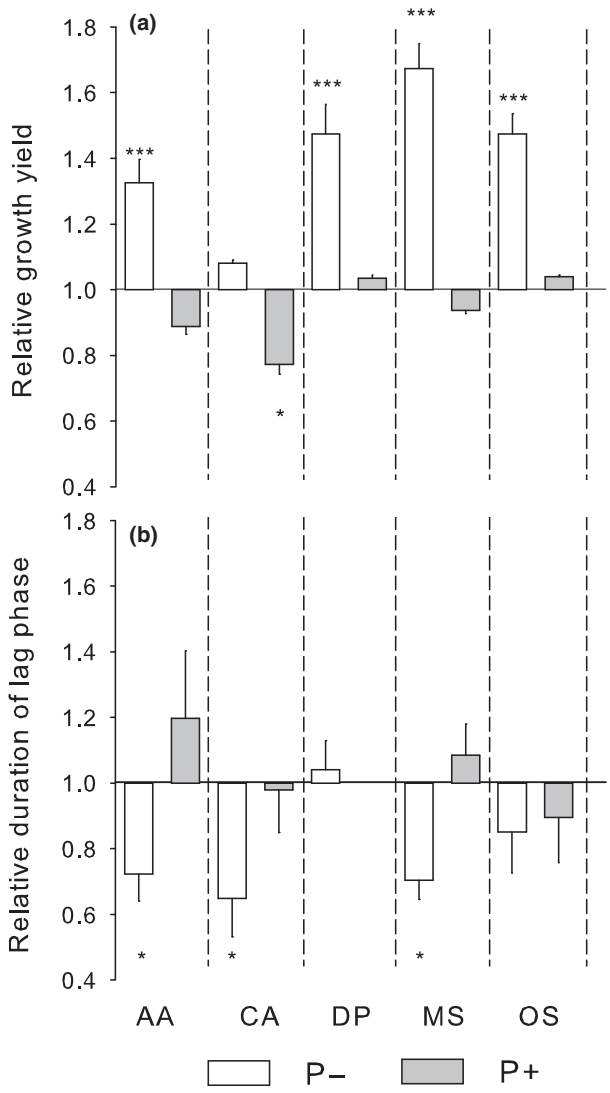

Fig. 6 Relative growth yield (a) or duration of the lag phase (b) (normalized to the ancestor) of evolved strains isolated after 26 growth cycles without (P−) or with (P+) predators on different substrate classes. $*P < 0.05$; $**P < 0.01$. AA, amino acids; CA carbolic acids; DP, dipeptides; MS, monosaccharides; OS, oligosaccharides.

microevolutionary adaptations at oligotrophic growth conditions may be beneficial for bacterial survival in the patchy landscape of the aquatic environment. A serial transfer of marine *Vibrio* isolates to liquid media with decreasing substrate concentrations (from g to mg C L^{-1}) also led to the dominance of strains with a 'facultative oligotrophic' life style that were able to grow well in both rich and poor media (Hood & Macdonell, 1987).

However, in some aspects, the P− isolates performed worse on rich medium than the ancestor: they had lower maximal growth rates and a prolonged lag phase (Fig. 5). While the latter can be beneficial under stressful condition, for example, during exposure to antibiotics (Fridman *et al.*, 2014), it may also lead to a competitive disadvantage: in cocultures of marine bacteria, an *Oceanospirillum* strain was outcompeted by a *Pseudoalteromonas* strain with shorter lag phase, even though *Oceanospirillum* could grow to substantially higher total densities in pure culture (Pernthaler *et al.*, 2001). Thus, coculture assays of the ancestor and the evolved *Sphingobium* strains would be required to resolve if the exposure to oligotrophy indeed resulted in improved competitive performance at the original growth conditions.

Interestingly, this apparent difficulty of the evolved strains to acclimatize to rich growth conditions was only observed on the original complex substrate mix. By contrast, when confronted with single carbon sources, the lag phase was either as long as that of the ancestor or even significantly shorter (Fig. 6b). This observation might be interpreted in the context of the model for facultative oligotrophic growth by Egli (Egli, 2010): Slowly growing *E. coli* cultivated on a single carbon source nevertheless maintained 'multivory', that is the ability to transport and metabolize a range of other substrates, and consequently their preparedness for substrate co-utilization and/or instantaneous switching. It is conceivable that the P− isolates also improved in their 'latent' ability to immediately utilize a variety of single C sources that were available in trace amounts in the complex medium. The closely related oligotrophy specialist *Sphingopyxis alaskensis* virtually showed no lag phase if slowly growing or starving cells were re-inoculated into glucose-rich medium (Eguchi *et al.*, 1996).

The observed broad upshift in substrate utilization efficiency (Fig. 6a) may not require the unlikely accumulation of several beneficial mutations. Suboptimal growth conditions induce a stress response in *E. coli* that is mediated by the alternative sigma factor RpoS. Differences in *rpo*S expression may profoundly affect bacterial carbon utilization patterns (King *et al.*, 2004). Growth in low substrate environments provides a positive selection pressure for mutations of *rpo*S (Ferenci, 2008), and phenotypic diversity in environmental *E. coli* isolates may arise solely by selection for such mutations (Chiang *et al.*, 2011). While *rpoS* itself is not found in the genomes of *Sphingobium* sp. strains, other regulatory circuits might work in the same direction. For example, *Sphingobium* spp. possess the ppGpp system, which is responsible for the stringent response upon carbon starvation (Atkinson *et al.*, 2011). A recent study indicates that the stringent response might be important in the *Sphingomonadacea* under limiting conditions: genes involved in this signalling cascade are up-regulated when *Sphingomonas* sp. are cultivated over a longer time period under nutrient-restricted conditions (Fida *et al.*, 2013).

Predation restricts adaptation to abiotic environment

The presence of predators in our experiment by far outweighed substrate limitation in determining the ratio of growth and defence morphotypes in the evolved strains (Fig. 1). The P+ treatment clearly selected for genotypes (strains) with constitutively enhanced aggregate and biofilm formation (Fig. 3). A prolonged cocultivation of *Serratia marcescens* and the ciliate *Tetrahymena thermophila* also pushed bacteria towards more grazing-resistant forms, both at low and high resource availability (Friman *et al.*, 2008). Such defence phenotypes are usually considered to represent a trade-off in terms of growth: the formation of both flocs and biofilms requires higher production of extracellular polymeric substances (Hahn *et al.*, 2004), for example polysaccharides (Yang & Kong, 2012) (Fig. 2), which is a particularly costly investment at substrate-limiting conditions. *Sphingobium* aggregates appeared to be of complex composition rather than the results of chance encounters of free cells; a variety of cellular morphotypes with contrasting surface properties could be distinguished by staining with fluorescently labelled lectins (Fig. 2). One might speculate that specific well-differentiated phenotypes, for example filamentous forms (Fig. 2d) or microcolonies (Fig. 2g–i), served as 'seeds' for flock formation, as has been described for aggregates composed of two species (Corno *et al.*, 2013). In any case, the aggregated phenotype probably represented a growth disadvantage and mainly functioned as a refuge from flagellate predation (Hahn *et al.*, 2004).

The overaggregating strains from the predator-exposed treatments had the longest growth delay and lowest OD_{600} at the original growth conditions (Fig. 5). Moreover, they were unable to metabolize a number of single substrates that were readily consumed by the ancestor and the P− strains (Table S2), and they had significantly lower growth yield on carbolic acids (Fig. 6). Our results agree with a previous report about the reduced resource use ability of a bacterial strain after co-evolution with predators at low substrate conditions (Friman *et al.*, 2008). The coevolution of bacteria and parasitic phages also had negative fitness costs for the hosts; it, moreover, restricted the number of beneficial mutations they could acquire to adapt to the abiotic environment (Scanlan *et al.*, 2015). The authors hypothesized that this might be due to negative epistasis between mutations that favoured growth and resistance, respectively. Increased mutation rates have also been reported to lead to a higher loss of metabolic functions and performance during long-term experimental evolution (Leiby & Marx, 2014). It is, however, unlikely that all isolates from the P+ treatment were hypermutators. Alternatively, the enhanced aggregation in P+ strains accompanied by the apparent loss of physiological functions might also be interpreted in terms of antagonistic pleiotropy: A *Pseudomonas fluorescens* genotype with superior fitness in a particular microniche (the air–water interface) had concomitant restrictions of carbon catabolism, and both phenomena were due to a mutation at the same locus (MacLean *et al.*, 2004).

The adaptation of *Sphingobium* sp. to predation interfered with the typical response of many bacteria to a low substrate environment. Specifically, it reduced the formation of motile free cells that are required at such conditions to search for the rare productive hotspots (Stocker *et al.*, 2008). This might be disadvantageous in some aquatic environments: Aggregation is a common mode of grazing protection in small eutrophic ponds or during productive periods such as phytoplankton blooms (Langenheder & Jürgens, 2001; Simon *et al.*, 2002). However, this growth type is virtually absent in oligotrophic lakes or the open ocean (Jürgens & Güde, 1994). Instead, these habitats are dominated by extremely small bacteria (Morris *et al.*, 2002; Salcher *et al.*, 2011) that have reduced encounter probabilities with predators (Shimeta, 1993) and that may be additionally protected by specific cell wall features (Tarao *et al.*, 2009). These entirely planktonic oligotrophy specialists have highly efficient substrate uptake systems (Sowell *et al.*, 2009), but are unable to grow at rich conditions (Rappe *et al.*, 2002). Thus, the costs of a specific predation defence strategy may play a role in defining the niches of aquatic bacteria.

Loss of physiological acclimation at constant selection conditions?

All evolved strains apparently lost their phenotypic plasticity with respect to predation defence: strains from the P+ treatment constitutively formed high numbers of aggregates in pure culture (Figs 3c and 4a), and the presence of predators did not induce this phenotype in P− strains (Fig. 4b). The response of the ancestor to predation was more plastic than that of the evolved strains: For one, the ratio of aggregates to free cells of the ancestor strain was approximately 40 times higher when grown with flagellates than in pure culture, but only between 5 and 10 times in the evolved strains (Fig. 4a,b). Secondly, the growth in supernatants of a spent predator–prey coculture led to enhanced aggregation in the ancestor, but not in the evolved strains (Fig. 4c).

Theoretical analyses propose that the 'specialist' genotype will always outcompete the plastic 'generalist' at constant growth conditions if there is a fitness cost to environmental perception and conditional physiological adaptation (Yamamichi *et al.*, 2011). As outlined above, growth conditions were probably not entirely constant in our experiments even in the absence of predators, and the somewhat delayed growth dynamics of

flagellates (as compared to bacteria, data not shown) suggest that this was even more the case for the P+ treatments. However, the propagation of bacteria from the P+ variants between individual growth cycles always took place when a large part of the total population was situated within aggregates (Fig. 1). One might thus envisage an interplay of ecological and evolutionary processes leading to the constitutive formation of aggregates in this phenotypically plastic population. First the aggregated phenotype is enriched by an ecological constraint (predation). At the same time, only very few cells are able to propagate over several generations in the free-living form. As a consequence, evolutionary processes would mainly occur within the numerically dominant aggregated subpopulation and would tend to favour adaptations that are beneficial to this phenotype.

Conclusions

We show that low substrate concentrations may lead to the loss of aggregation and biofilm formation in a bacterial strain and improve its performance on a variety of single carbon sources. However, concomitant predation may prevent this evolutionary trajectory and lead to the constitutive overexpression of grazing-protected growth forms. Thus, predation mortality (in combination with patchy substrate availability) may play an important role in co-determining the balance between free and attached cells in bacterial taxa with a dual life style. Moreover, the instability of these selective factors might be responsible for the ability of bacterial populations to conditionally shift between growth forms. It remains to be investigated whether phenotypic plasticity is preserved in evolving *Sphingobium* sp. Z007 if maintained as suggested by theory (Yamamichi *et al.*, 2011), that is, at more drastic fluctuations of top-down and bottom-up control. This could be experimentally realized by exposing bacteria to the predators only during every second growth cycle.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 Lectins that showed strong or weak binding signal to the matrix or surface structures of a *Sphingobium* sp. Z007 strain evolved with flagellates for 16 growth cycles.

Table S2 Consumption patterns of single carbon sources by the *Sphingobium* sp. strain Z007 ancestor, and by 3 isolates from replicate vessels evolved without (EvoA, B, C) or with (EvoPA, PB, PC) the bacterivorous flagellate *Poterioochromonas* sp. strain DS.

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