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ORIGINAL ARTICLE

Disentangling the relative influence of bacterioplankton phylogeny and metabolism on lysogeny in reservoirs and lagoons

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Previous studies indicate that lysogeny is preponderant when environmental conditions are challenging for the bacterial communities and when their metabolism is reduced. Furthermore, it appears that lysogeny is more frequent within certain bacterial phylogenetic groups. In this comparative study from 10 freshwater reservoirs and 10 coastal lagoons, we aim to disentangle the influence of these different factors. In eight reservoirs and four lagoons, lysogeny was detected by induction assays with mitomycin C, and induction significantly modified the bacterial community composition (BCC), whereas community composition remained constant in ecosystems in which lysogeny was not observed. Among the phylogenetic groups studied, the most abundant ones were Bacteroidetes and α-proteobacteria in lagoons, and β-proteobacteria and Bacteroidetes in reservoirs. These dominant groups comprised the highest proportions of inducible lysogens. In order to unravel the effects of bacterial metabolism from phylogeny on lysogeny, we measured bacterial community physiology and the specific activities of selected phylogenetic groups. The proportion of inducible lysogens within the α- and the β-proteobacteria decreased with increasing group-specific metabolism in lagoons and reservoirs, respectively. In contrast, this relationship was not observed for the other lysogen-containing groups. Hence, both host physiology and phylogeny are critical for the establishment of lysogeny. This study illustrates the importance of lysogeny among the most abundant phylogenetic groups, and further suggests its strong structuring impact on BCC.
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Introduction

Bacteriophages are considered to be the most abundant and diversified component of aquatic ecosystems, in which they hold major functional roles (Fuhrman, 1999; Weinbauer, 2004). They replicate via two main cycles, the lytic and the lysogenic cycle (Ackermann and DuBow, 1987; Weinbauer, 2004). During the lytic cycle, viral infection leads to the direct production of new viral particles and host lysis. During the lysogenic cycle, the viral genome integrates into the host's replicon and remains as a prophage within the bacterium called a lysogen. This prophage will remain in the lysogen until induction takes place, triggering a shift to the lytic cycle and the production of new phages in the system. Numerous studies have indicated that lysogeny could be a survival strategy for the virus, in which the host would constitute a refuge under challenging environmental conditions for the bacterial communities, and unfavorable conditions for lytic replication (McDaniel *et al*., 2002; Weinbauer *et al*., 2003; Paul, 2008).

Studies on the effect of viral infection on bacterial diversity have mostly focused on lytic infection, and are based on the assumption that viral lysis shapes the bacterial community composition (BCC) as predicted by the 'killing the winner' hypothesis (Thingstad and Lignell, 1997). Recent studies within the frame of this hypothesis have further indicated that the dominant bacteria are often resistant to viral lysis (Suttle, 2007; Middelboe *et al*., 2009). This resistance can result from the endless arms race between a phage and its host or from acquired

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immunity (Suttle, 2007; Rohwer and Vega Thurber, 2009).

Lysogens are homo-immune to infection of related phages, and can even expand their distribution by acquiring ecologically relevant genes (Ackermann and DuBow, 1987; Rohwer and Vega Thurber, 2009). Data on the effects of lysogenic infections on natural BCC are still sparse, but indicate two different results. Lysogens can affect community structure by increasing their abundance via their acquired immunity, but their existence in the system is highly dependent on the absence of induction events (Paul, 2008). Hence, temperate phages could also strongly shape the bacterial community structure when induction occurs, as suggested earlier by Hewson and Fuhrman (2007). BCC differs within aquatic ecosystems, generally with a dominance of β-proteobacteria in freshwater systems (Glöckner *et al.*, 1999; Rappé *et al.*, 2000), and Bacteroidetes and α-proteobacteria in lagoons (Benlloch *et al.*, 1996; de Wit, 2008). In addition, bacterial phylogenetic groups show large differences in their specific activity (del Giorgio and Gasol, 2008). Yet to date, it is unclear whether larger phylogenetic groups, sharing common physiological and metabolic characteristics (Cottrell and Kirchman, 2000; Yokokawa *et al.*, 2004; Bühring *et al.*, 2005), may be collectively more affected than others by lysogeny.

Genomic sequencing data have highlighted the importance of lysogeny for aquatic bacteria, with 60 to 70% of bacterial genomes sequenced to date estimated to contain prophages (Casjens, 2003; Srividhya *et al.*, 2007; Paul, 2008). Furthermore, lysogeny has been previously suggested to be more widespread in certain phylogenetic groups, such as the γ-proteobacteria, the Gram-positive bacteria and some taxa of the SAR11 clade within the α-proteobacteria (Brüssow *et al.*, 2004; Hewson and Fuhrman, 2007). However, these studies have not considered bacterial metabolism, and none have separated the role of bacterial metabolism from that of bacterial phylogeny on the occurrence of lysogeny.

In order to understand the mechanisms of lysogeny at the single-cell level, we measured a set of variables exploring whether major phylogenetic groups differ in their susceptibility to lysogenic infection, and whether bacterial metabolism (assessed by physiological proxies and radiolabelled substrate uptake) is also implicated. We sampled 10 distinct sites in two types of ecosystems, freshwater reservoirs and coastal lagoons. This allowed us to screen selected bacterial phylogenetic groups within contrasting settings and with different characteristics, to reveal the possible links between phylogeny, metabolism and lysogeny. Bacterial physiology and community metabolism, as well as the specific activity of five selected phylogenetic groups, were analyzed at the single-cell level.

The specific aims of this study were to detect within natural communities from fresh and coastal lagoon waters, whether (i) some of the selected bacterial phylogenetic groups contain more inducible lysogens than others, and whether this is consistent throughout both types of systems; (ii) the patterns between the bacterial groups and lysogeny are solely due to phylogenetic affiliation, or to other bacterial features; such as metabolism, or to environmental factors; and (iii) induction is a strong factor shaping the BCC.

Materials and methods

Sampling

In total, 10 coastal lagoons and 10 freshwater reservoirs from the south of France, all listed in Table 1, were sampled in June 2008. These relatively shallow lagoons (mean depth: 1.5 m) were selected to obtain a wide range of salinities (from 11.1 to 37.7) and chlorophyll *a* values (from 0.2 to 9.3 $\mu g\,l^{-1}$; see Bec, 2005), whereas the reservoirs were selected on geographic criteria, as no limnological data existed for these systems. Water was sampled at the surface ranging from 0.5 to 1 m, using acid-rinsed polycarbonate bottles thoroughly rinsed with milli-Q water. Samples were kept cool and stored in darkness for less than 4 h before processing. Temperature, salinity, pH and oxygen measurements were measured directly on site with a multiparameter Multi 350i instrument (WTW, Weilheim, Germany).

Environmental and chemical variables

Triplicate 50-ml samples were filtered on GF/F filters for determination of chlorophyll *a* concentration, according to Yensch and Menzel, 1963, using a Trilogy fluorometer (Turner, Sunnyvale, CA, USA) after extraction in 90% acetone. To assess total nitrogen and phosphorous concentrations, respectively, triplicate samples were oxidized with persulfate before being analyzed with a Technicon Autoanalyzer AA3 (Menzel and Corwin, 1965; Raimbault and Slawyk, 1991).

Viral enumeration

Viral abundance was determined after nucleic acid staining with SYBRGold (Invitrogen, Carlsbad, CA, USA) on 0.02-μm pore size Anodisc-membranes (Whatman, Maidstone, UK) by epifluorescence microscopy, following Chen *et al.* (2001). Viruses were then enumerated with an Olympus Provis-AX70 epifluorescence microscope. Fixation and filtration took place directly after the sampling, and filters were kept at –20 °C. Staining and mounting for epifluorescence observations took place within a week of storage. A minimum of 20 fields and 400 viruses per filter was counted.

Bacterial enumeration and single-cell characteristics

Total bacterial abundance and the proportion of respiring cells were obtained from fresh, unfixed

Table 1 Average values of environmental and biological parameters

Ecosystem	*Geographic coordinates*	*Temp (°C)*	*pH*	*O₂ (% satur.)*	*Chl a (μg l⁻¹)*	*TP (μmol l⁻¹)*	*TN (μmol l⁻¹)*	*Salinity*	*Virus (× 10⁸ ml⁻¹)*	*Bacteria (× 10⁶ ml⁻¹)*	*VBR*	*% CTC⁺*
Freshwater reservoir		*21.6 (1.7)*	*8.3 (0.4)*	*96.7 (11.9)*	*4.2 (4.9)*	*0.8 (0.7)*	*48.4 (27.1)*	*0.0 (0.0)*	*1.6 (0.9)*	*4.6 (2.2)*	*36.5 (11.7)*	*39.7 (31.2)*
Villeveyrac	+43°29′19″N, +03°36′49″E	20.5	9.2	96.3	2.7 (0.5)	0.9 (0.0)	40.8 (4.1)	0.0	1.6 (0.2)	4.1 (0.0)	39.9	96.8 (4.2)
Les Olivettes	+43°33′32″N, +3°17′52″E	19.8	8.7	94.6	3.3 (1.9)	0.7 (0.0)	58.4 (8.5)	0.0	2.9 (0.3)	5.8 (0.5)	49.7	42.6 (4.0)
Salagou	+43°39′24″N, +3°22′16″E	19.6	8.5	95.2	4.4 (0.4)	1.0 (0.1)	42.4 (4.2)	0.0	2.0 (0.2)	5.9 (0.2)	34.2	42.3 (1.6)
La Jasse	+43°46′57″N, +3°46′30″E	20.7	7.9	83.6	0.8 (0.3)	0.3 (0.1)	22.8 (9.6)	0.0	0.8 (0.1)	1.8 (0.1)	42.5	71.3 (2.9)
Le Crès	+43°39′18″N, +3°55′84″E	22.8	8.4	102.6	0.9 (0.1)	0.5 (0.0)	42.1 (2.8)	0.0	1.1 (0.1)	6.7 (0.9)	16.4	15.6 (2.0)
Cécèles	+43°45′49″N, +3°53′21″E	21.8	8.0	90.3	2.3 (0.4)	0.3 (0.0)	29.2 (3.1)	0.0	0.6 (0.1)	1.4 (0.0)	43.6	2.7 (2.5)
Tréviers	+43°46′44″N, +3°52′11″E	23.0	8.3	83.0	18.6 (2.6)	2.7 (0.2)	64.4 (11.2)	0.0	3.3 (0.3)	6.2 (0.1)	53.6	65.9 (8.3)
Claret	+43°51′33″N, +3°52′29″E	21.3	7.9	88.8	1.7 (1.2)	0.4 (0.2)	14.7 (0.5)	0.0	0.6 (0.0)	2.8 (0.1)	22.4	3.7 (3.9)
La Rouvière	+43°55′55″N, +4°01′26″E	20.9	7.8	107.6	4.7 (0.4)	0.7 (0.0)	116.6 (5.9)	0.0	1.1 (0.1)	3.3 (0.4)	32.1	13.5 (1.0)
Fréjorgues	+43°36′23″N, +3°57′26″E	25.5	8.3	125	3.1 (0.2)	0.7 (0.0)	53.1 (0.1)	0.0	2.4 (0.1)	7.7 (0.2)	30.7	18.7 (2.2)
Coastal lagoon		*20.4 (1.0)*	*8.5 (0.3)*	*84.7 (17.8)*	*2.3 (2.9)*	*2.2 (1.4)*	*48.9 (16.4)*	*24.6 (8.8)*	*3.4 (1.2)*	*8.3 (2.9)*	*43.1 (14.6)*	*16.7 (9.0)*
Salses-Leucate	+42°°50′20″N, +2°59′33″E	19.3	8.3	71.8	0.7 (0.1)	0.7 (0.0)	32.4 (0.9)	37.7	2.3 (0.1)	14.0 (0.2)	16.4	29.3 (1.1)
Bages-Sigean	+43°07′53″N, +2°59′57″E	19.2	8.7	78.9	0.6 (0.1)	1.1 (0.0)	32.8 (0.4)	33.3	2.8 (0.3)	10.5 (0.3)	26.5	17.0 (1.1)
Campignol	+43°06′01″N, +3°02′41″E	19.9	8.2	62.0	9.3 (2.5)	3.7 (0.0)	64.4 (0.6)	13.2	2.5 (0.3)	5.2 (0.6)	46.9	15.1 (0.5)
Marseillan	+43°21′12″N, +3°33′24″E	20.2	8.1	71.4	6.2 (1.7)	2.2 (0.1)	53.8 (0.6)	33.3	3.8 (0.2)	8.6 (0.4)	43.9	21.3 (0.1)
Thau	+43°26′09″N, +3°39′24″E	19.3	8.2	90.2	1.2 (0.8)	1.1 (0.0)	26.4 (0.2)	33.6	2.5 (0.3)	4.8 (0.0)	51.9	34.1 (0.5)
Ponant	+43°33′52″N, +4°06′52″E	20.9	8.1	97.0	2.5 (1.0)	2.6 (0.0)	80.2 (0.7)	11.1	2.0 (0.2)	6.2 (0.3)	33.1	8.4 (0.5)
Or	+43°34′42″N, +4°02′05″E	20.7	8.4	128.0	0.9 (0.4)	1.4 (0.1)	51.4 (1.8)	18.9	4.6 (0.5)	10.1 (0.5)	45.4	7.8 (0.3)
Grec	+43°32′16″N, +3°56′38″E	21.1	9.2	70.5	0.2 (0.1)	1.9 (0.0)	57.6 (0.1)	19.5	6.0 (0.3)	10.0 (0.1)	59.8	5.1 (0.3)
Prévost	+43°31′22″N, +3°54′16″E	20.5	8.6	88.5	0.3 (0.1)	1.1 (0.0)	33.0 (0.1)	22.3	3.0 (0.3)	7.0 (0.1)	42.5	19.2 (0.4)
Méjean	+43°33′02″N, +3°55′01″E	22.5	8.9	88.4	1.1 (0.4)	5.7 (0.0)	57.1 (0.2)	23.1	4.8 (0.6)	6.8 (0.3)	64.7	9.9 (1.1)

Abbreviations: Chl *a*, chlorophyll-*a* concentrations; CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; O_2, percent saturation of oxygen; Temp, temperature; TP and TN, total phosphate and nitrogen concentrations.

Mean chemical variables, bacterial and viral abundances, the resulting virus-to-bacteria ratio and the proportion of respiring CTC^+ cells within each ecosystem are provided. For each type of ecosystem (freshwater reservoirs vs lagoons), general mean values and s.d. (in parenthesis) are also given. When possible (e.g., for parameters measured in triplicate), specific s.d. values (in parenthesis) are given.

samples, using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Total bacterial abundance was determined after nucleic acid staining with SYBRGreenI (Invitrogen). Stained bacteria were discriminated and enumerated according to del Giorgio *et al.* (1996). The abundance of respiring bacteria with high rates of metabolism was determined using the 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, tebu-bio SAS), an indicator of the respiratory electron transport system activity (see procedure in Sherr *et al*., 1999). The percentage of respiring CTC^+ cells was calculated relative to the total bacterial counts obtained by SYBRGreenI staining and flow cytometer analysis, and was considered as a proxy of actively growing cells (del Giorgio and Gasol, 2008).

Frequency of inducible lysogens
The proportion of inducible lysogens, classically referred to as the frequency of lysogenic cells (FLC), was obtained with triplicate incubations of untreated samples (control incubations) and samples treated with mitomycin C ($1\,\mu g\,ml^{-1}$ final concentration; Sigma, St Louis, MO, USA). This antibiotic is a toxic antineoplastic agent and a potent DNA cross-linker agent, preventing DNA synthesis (Windholz *et al*., 1976; Pradeep Ram and Sime-Ngando, 2009). This results in prophage induction by direct DNA damage (Iyer and Syzbalski, 1963). All the ecosystems sampled were alkaline, precluding the instability of this antibiotic under acidic conditions (Windholz *et al*., 1976). During preliminary 24-h time series incubations, we observed that incubations longer than 17 h were unnecessary. Three subsamples (6, 8 and 17 h) of these incubations were fixed with formaldehyde (2% final concentration for bacterial and viral enumeration by SYBRGold staining as above. Induction was detected when we observed simultaneously a significantly lower bacterial abundance and higher viral abundance in the mitomycin C treatment relative to control (Cochran and Paul, 1998; Tapper and Hicks, 1998). Both changes in abundance argue for the detection of inducible lysogens. FLC was calculated according to the equation published by Weinbauer *et al*. (2003). We used average published minimum (FLC_{minBS}) and maximum (FLC_{maxBS}) burst size values adequate for each type of ecosystem (37 and 53 for the freshwater reservoirs, and 24 and 35 for the lagoons, respectively; Parada *et al*., 2006). Maximum burst size was obtained using the equation 'maximum burst size = 1.41 minimum burst size + 0.87' (Parada *et al*., 2006).

Lysogeny and BCC
The BCC was obtained by catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) with group-specific oligonucleotide probes on membrane filters following Pernthaler *et al*. (2004). Seven horseradish peroxidase probes (Biomers, Ulm, Germany) targeted the domain Bacteria (EUB338 I-III mix), the α-, β- and γ-proteobacteria (ALF968, BET42a and GAM42a), the Bacteroidetes (CF319a) and the SAR11 clade. After the hybridization procedure, filters were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) ($1\,\mu g\,ml^{-1}$; Euromedex, Mundolsheim, France) and the proportions of hybridized cells were counted using an Olympus Provis-AX70 epifluorescence microscope. The error associated with replicate CARD-FISH counts ranged from 3 to 8% (mean 5%), based on a subset of 10 samples for which we conducted independent replicate CARD-FISH counts. The error on the percentage of bacteria was 14%, and corresponded to the sum of errors for the bacterial group counts and for total bacterial counts (average 4%), according to standard propagation of error equations. This error was consequently applied to all CARD-FISH analyses. The % EUB was also considered as a proxy of bacterial activity (Bouvier and del Giorgio, 2003).

To determine whether certain bacterial phylogenetic groups contain inducible lysogens, CARD-FISH analyses were made on both control and mitomycin C-added samples. For each bacterial group, we calculated the change occurring because of the addition of mitomycin C with the equation:

$$\Delta_P = 100 \times (N_{P,M} - N_{P,C})/D_C \quad (1)$$

in which Δ_P, expressed as a percentage, represents the difference between the mitomycin C-amended treatment and the control sample for the phylogenetic group *P*; $N_{P,M}$, the number of bacteria from the phylogenetic group *P* in the mitomycin C-amended treatment; $N_{P,C}$, the number of bacteria from the phylogenetic group *P* in the control sample; and D_C, the number of DAPI cells in the control sample. With this equation, changes in the proportion of a given bacterial group reflect changes in the abundance of these bacteria, and do not result from variations in the proportions of the other phylogenetic groups. Hence, a negative Δ_P shows that the number of bacteria belonging to the specific group *P* has decreased in the mitomycin C treatment relative to the control, indicating that this group contained a significant proportion of inducible lysogens. Using Δ_P values for inferring lysogeny in the different groups is thus based on the assumption that each bacterial phylogenetic group will grow and be grazed with the same group-specific rates both in the control and in the treatment sample, regardless of the treatment effect. In principle, such ecological interactions may interfere with the use of Δ_P values for inferring lysogeny, but this impact is presumed to be low in the short-term assays. These analyses were made in samples in which induction was detected, at the corresponding time point (T6, T8 or T17). However, we also determined the Δ_P of each bacterial group in samples without detected induction at the end of the incubations (T17), particularly to determine the antibiotic effect of mitomycin C on

BCC, which could interfere with the interpretation of the Δ_P values. The comparison of the different Δ_P using appropriate statistical analyses allowed us to determine the impact of induction on each selected bacterial phylogenetic group.

Bacterial specific activity

Microautoradiography combined with CARD-FISH (MAR-FISH) allows the identification of active substrate-uptaking cells. Leucine is an amino acid incorporated by the majority of actively growing cells (Kirchman *et al*., 1985). Within each specific bacterial group, the proportion of cells actively incorporating leucine can be considered a proxy of the average *in situ* activity of the group. We followed the protocol described by Alonso and Pernthaler (2005), using the previously mentioned oligonucleotide probes. Briefly, killed controls (2% formaldehyde fixation) and samples were incubated for 1 h at *in situ* temperature with ^{3}H-Leucine (40 nM final concentration), before fixation (formaldehyde, 2% final concentration) and filtration. We then proceeded with the CARD-FISH protocol, carrying out the supplementary dipping in photographic emulsion and revealing steps in a dark room before the final DAPI counterstaining (Alonso and Pernthaler, 2005).

Statistical analyses

Statistical analyses were carried out using the JMP 5.01 (SAS Inc., Cary, NC, USA) and R software (http://www.r-project.org/). One-way analysis of variance was performed between control and treatment samples at each time point to determine whether induction occurred. All data were checked for normality. To explore the impact of induction, we studied how Δ_P could be explained by the set of environmental parameters, bacterial metabolic features and by FLC values, using a variance partitioning approach (Borcard *et al*., 1992; Peres-Neto *et al*., 2006). We used a combination of redundancy analyses and partial redundancy analyses (Legendre and Legendre, 1998) to determine how much the different effects can independently explain variations in Δ_P. We thus extracted the pure effect of each set of explanatory variables, as well as their combined effects on Δ_P values.

Second, we tested the effects of all variables, including the phylogenetic groups, on Δ_P in the samples with induction. To this aim, we used two multiple linear regressions to relate Δ_P values to all the potential explanatory variables, for each type of ecosystem. Explanatory variables were then selected by a backward stepwise elimination procedure from the general model according to the Akaike criterion (Burnham and Anderson, 2002). The most parsimonious model is the one with the lowest Akaike criterion, and was selected as the best-fit model out of all the models under consideration.

Results

Environmental and biological parameters

Mean values of all environmental and biological parameters measured within each ecosystem are shown in Table 1, with their s.d. values. Temperature, chlorophyll *a*, total nitrogen and oxygen concentration were widely variable between both environment types, and were thus not statistically different (Tukey-Kramer *post hoc* analysis, $P > 0.05$), whereas total phosphorous concentrations were significantly higher in lagoons (Table 1).

Bacterial abundance in lagoons averaged 8.3×10^6 cells per ml and 4.6×10^6 cells per ml in freshwater reservoirs. Mean viral abundance in lagoons was 3.4×10^8 viruses per ml and 1.6×10^8 viruses per ml in freshwater reservoirs. Both abundances were significantly higher in coastal lagoons than in reservoirs ($P < 0.05$). Resulting virus-to-bacteria ratios were similar between ecosystems types ($P > 0.05$), ranging from 16.4 to 53.6 in reservoirs and from 16.4 to 64.7 in lagoons (Table 1). The average proportion of respiring cells (CTC$^+$ cells) was higher in the freshwater reservoirs (39.7%), but this was not statistically different from the lagoons (16.7%), due to the high variability of values (Table 1).

The probe complementary to a region of the 16S rRNA conserved in most Bacteria (EUB) detected on average 43% (reservoirs) and 69% (lagoons) of the *in situ* total number of cells determined by DAPI direct counts. Thus, on average, 57% and 31% of the prokaryotes were unaccounted by the set of probes we used. The probes for α-, β- and γ-proteobacteria and the Bacteroidetes together accounted for an average of 72% (reservoirs) and 90% (lagoons) of the cells hybridized with the universal Bacterial probes (EUB338 I-III mix). The set of probes together allowed a clear detection of differences in the BCC between ecosystem types (Table 2). The β-proteobacteria and Bacteroidetes were dominant among the selected groups in the BCC in freshwater reservoirs, constituting on average 19.3 % and 11.3% of the total community, respectively. In contrast, in lagoons, the α-proteobacteria and Bacteroidetes accounted on average for 30% and 22.6% of the total community, respectively (Table 2).

The MAR-FISH experiments revealed the proportion of cells actively uptaking leucine for each selected phylogenetic group (Table 2). In lagoons, the α-proteobacteria was the most active group with on an average 54.2% of cells that were active, followed by the γ- and β-proteobacteria (with 45.3% and 43.7% of cells that were active, respectively). In reservoirs, the β- and α-proteobacteria were the most active groups with on average 61.9% and 51.9% of cells actively taking up the substrate, respectively, whereas 39.6% of the γ-proteobacteria were active. The Bacteroidetes were generally the least active cells in lagoons (on average 39% of cells were active) and reservoirs (on average 41% of cells were active) (Table 2). Overall, there was important variability of

Table 2 Bacterial phylogenetic groups abundance and specific activity in both types of ecosystems

Bacterial phylogenetic group	*In situ abundance (%), average (s.d.)*	*Tukey-Kramer analysis*	*^{3}H-Leucine-assimilating cells (%), average (s.d.)*	*Tukey-Kramer analysis*
Freshwater reservoirs				
α-proteobacteria	10.8 (13.2)	BC	51.9 (11.2)	AB
β-proteobacteria	19.3 (14)	A	61.9 (21.3)	A
γ-proteobacteria	3.8 (3.4)	BC	39.6 (14.4)	C
Bacteroidetes	11.3 (10.7)	AB	41.0 (13.2)	BC
SAR 11	0.4 (0.4)	C	42.6 (14.0)	BC
Coastal lagoons				
α-proteobacteria	22.6 (12.9)	AB	54.2 (14.0)	A
β-proteobacteria	3.1 (3.9)	D	43.7 (11.0)	B
γ-proteobacteria	13.8 (9.5)	BC	45.3 (3.9)	AB
Bacteroidetes	30.0 (20.9)	A	39.0 (6.8)	B
SAR 11	9.9 (9.9)	CD	46 (11.4)	AB

Abbreviation: DAPI, 4′-6-diamidino-2-phenylindole.
Cells from the five selected groups were counted and expressed as a percentage of the total DAPI-stained bacterial cells. The results from Tukey-Kramer *post hoc* analyses are indicated; within each type of ecosystem, mean percentages not connected by the same letter are significantly different ($P<0.05$).

bacterial metabolism and physiology between and within ecosystem types (Tables 1 and 2).

Occurrence of lysogeny and FLC

Lysogeny was encountered in 60% of the 20 sampled ecosystems (four lagoons and eight freshwater reservoirs). In these 12 systems, FLC_{minBS} showed comparable mean values between lagoons and freshwater reservoirs, with 32.3 and 36.5%, respectively (Table 3). A similar pattern was observed with the average FLC_{maxBS} in the lagoons and in the reservoirs, with 22.4 and 25.5%, respectively. Freshwater reservoirs for their part showed a higher incidence of lysogeny and wider range of values (range of FLC_{minBS}: 7.6–82.9%; range of FLC_{maxBS}: 5.3–57.9%) than the lagoons (range of FLC_{minBS}: 24.2–51.8%; range of FLC_{maxBS}: 16.8–35.8%). FLC negatively correlated with the percent of total hybridized cells (% EUB) in lagoons, and with the proportion of respiring cells (% CTC^{+}) in reservoirs, whether considering FLC_{minBS} or FLC_{maxBS} (Figure 1). No other biotic or abiotic parameter correlated significantly with FLC (data not shown).

Mitomycin C incubations and changes in BCC

The proportion of hybridized bacterial cells % EUB within control and mitomycin C incubations averaged 73.6 and 68.3%, respectively, with no significant difference between both samples. In samples without detected induction, we observed that the effect of mitomycin C on BCC was very limited, with average Δ_P values generally ranging between –5 and 0% (Figure 2a). The β-proteobacteria seemed to be the most sensitive group, with an invariably negative Δ_P (–2.3% on average). No consistent trends were observed for the other groups. Additionally, in these incubations with no detected induction, the effect of mitomycin C did not change the dominance patterns of the selected phylogenetic groups (Figures 3a and b).

In contrast, in samples in which induction was detected, the changes in BCC were much more

Table 3 Proportions of lysogenic cells (FLC) within each sampled ecosystem, expressed as FLC_{minBS} and FLC_{maxBS} that were calculated using literature values for the minimum and the maximum burst sizes, respectively (see Materials and methods section)

	% FLC	
Ecosystem	*FLC_{minBS}*	*FLC_{maxBS}*
Freshwater reservoir	*36.5 (12.3)*	*25.5 (19.6)*
Villeveyrac	38.9	27.2
Les Olivettes	ND	ND
Salagou	24.5	17.1
La Jasse	13.0	9.1
Le Crès	9.6	6.7
Cécèles	7.6	5.3
Tréviers	47.2	32.9
Claret	ND	ND
La Rouvière	68.4	47.8
Fréjorgues	82.9	57.9
Coastal lagoon	32.3 (8.7)	22.4 (9.0)
Salses-Leucate	ND	ND
Bages-Sigean	27.5	19.0
Campignol	ND	ND
Marseillan	ND	ND
Thau	ND	ND
Ponant	25.8	17.9
Or	ND	ND
Grec	24.2	16.8
Prévost	ND	ND
Méjean	51.8	35.8

Abbreviations: FLC, frequency of lysogenic cells; ND, not detected.
Average values for both types of ecosystems (freshwater reservoirs vs lagoons), and s.d. values (in parenthesis) are also given.

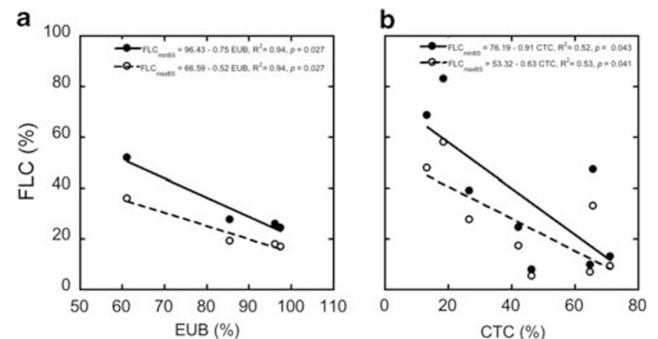

Figure 1 Linear correlations between the frequency of lysogenic cells calculated from minimum (black circle, FLC_{minBS}) and maximum (open circle, FLC_{maxBS}) burst size and (**a**) the proportion of Bacteria (% EUB) in coastal lagoons, and (**b**) the proportion of respiring bacterial cells (% CTC^{+}) in reservoirs.

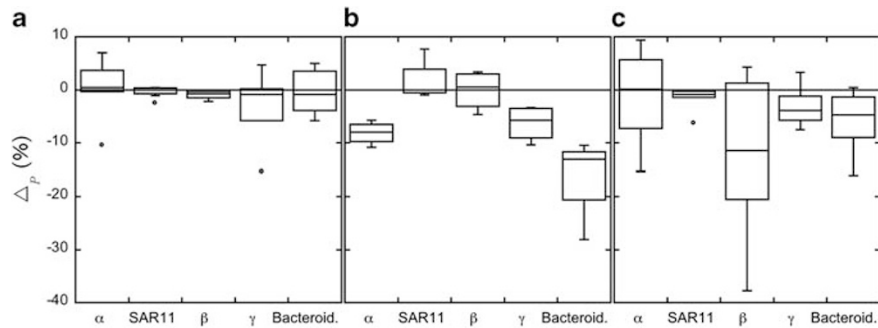

Figure 2 Box plots of the Δ_P of each bacterial phylogenetic group (see Materials and methods for Δ_P calculation) in (**a**) ecosystems without detected induction, (**b**) coastal lagoons with induction and (**c**) freshwater reservoirs with induction. Each box comprises the two middle quartiles, separated by the median. The whiskers represent 1.5 times the inner quartile spread in length, and provide an arbitrary cut-off point to identify data points that possibly are outside values. The small circles denote outlier values.

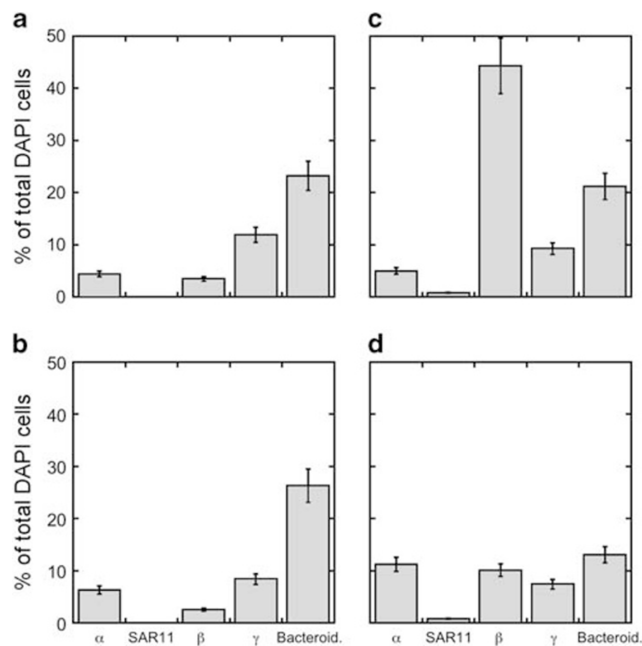

Figure 3 Example of bacterial community composition changes in a system without induction (**a**, **b**), Campignol lagoon and with induction (**c**, **d**, Fréjorgues reservoir). (**a**, **c**) Are control samples, and (**b**, **d**) are mitomycin C-amended incubations. Data are expressed as percentages of total DAPI counts. Error bars were determined from a subset of replicated samples (see Materials and methods).

important (with average Δ_P ranging from –38 to 9%, see Figures 2b and c). This resulted in drastic changes of the dominance patterns of the phylogenetic groups (Figures 3c and d). The partition analysis indicated that the environmental parameters, bacterial features and FLC_{minBS} explained 11, 9.3 and 15.3%, respectively, of the observed changes in Δ_P. Thus, FLC was the most significant explanatory factor for the changes in Δ_P, statistically confirming that changes in BCC (assessed by the Δ_P) when induction takes place contrast from those in samples without induction. The same conclusions were obtained with FLC_{maxBS} (data not shown). This statistical approach unambiguously shows that some phylogenetic groups comprised more inducible lysogens than others. In particular, in the lagoons where lysogeny was detected (Figure 2b), the Bacteroidetes, the α-, and to a lesser extent, the γ-proteobacteria were negatively affected (Δ_P= –16.1, –8.1 and –6.2%, respectively). The SAR 11 (Δ_P = 1.7%) and the β-proteobacteria (Δ_P = −0.05%) were virtually not affected. Both FLC_{minBS} and FLC_{maxBS} correlated with the Δ_P of Bacteroidetes ($R^2 > 0.90$, $P < 0.03$), with higher values of FLC corresponding to more negative values of Δ_P of Bacteroidetes. In the freshwater reservoirs with detected lysogeny (Figure 2c), induction affected most often the β-proteobacteria and the Bacteroidetes (Δ_P = –8.7 and –5.7%, respectively), followed by the γ-proteobacteria (Δ_P = –1.6%), the SAR 11 (Δ_P = –1.4%) and the α-proteobacteria (Δ_P = –1.1%). In these systems, FLC_{minBS} and FLC_{maxBS} correlated with the Δ_P of both the Bacteroidetes and the β-proteobacteria ($R^2 > 0.50$, $P < 0.05$; $R^2 > 0.60$, $P < 0.03$, respectively). The other groups did not show any pattern. Thus, induction had a different impact on the five bacterial phylogenetic groups, and dissimilar patterns between the types of ecosystems were observed.

Relationships between induction and bacterial metabolism within the different phylogenetic groups

Correlations between the specific activity of the phylogenetic groups and their Δ_P are presented in Figure 4. For the α-proteobacteria in lagoons, their Δ_P showed a significant positive correlation with the proportion of actively leucine-uptaking cells. In the reservoirs, the same significant and positive correlation was found within the β-proteobacteria. The Bacteroidetes showed the same trends, although not significant, in both types of ecosystems. No clear trends were detected for the other groups.

Disentangling the variables' effects on induction-based changes in Δ_P

Using two multiple regressions, the phylogenetic group and the activity of the bacterial community,

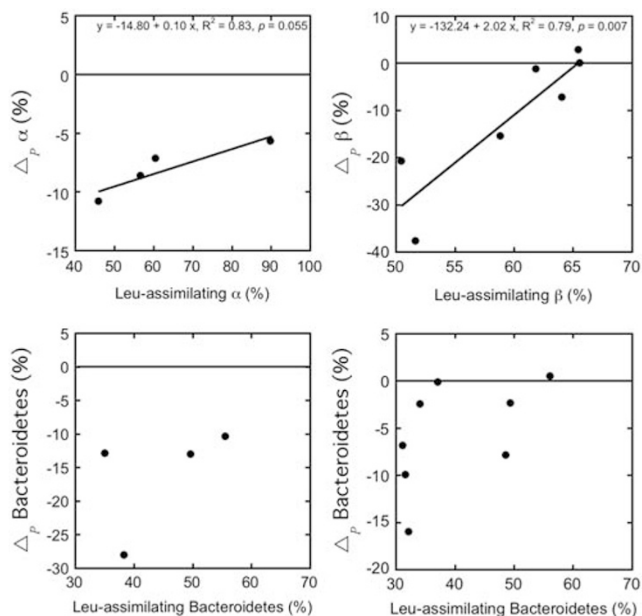

Figure 4 Linear correlations between the proportions of leucine-assimilating bacterial cells and the Δ_P values within three different bacterial phylogenetic groups. Left panel: coastal lagoons. Right panel: freshwater reservoirs.

assessed by the proportion of respiring cells, were found to be significant explanatory variables for the changes in the Δ_P (accounting for 3 and 13%, respectively, of the changes, $P<0.05$). Environmental parameters were not retained in the most parsimonious model and did not directly explain changes in Δ_P.

Discussion

In the 20 studied systems encompassing a wide range of environmental conditions, lysogeny was detected in more than half of the sites. In these 12 cases, induction significantly changed the BCC, as discussed hereafter. Lysogeny was not measurable in the remaining eight systems, which however does not necessarily imply an absence of lysogeny in these systems. Hence, lysogens could be temporarily in low abundance after a recent natural induction event, such as salinity fluctuations (Shkilnyj and Koudelka, 2007) or high solar radiation (Weinbauer and Suttle, 1999; Bettarel *et al.*, 2008). This might be particularly true for the lagoons ecosystems, in which detectable induction only occurred in 4 out of 10 sites, and which are intrinsically characterized by large variations in salt concentration. Alternatively, lysogens could be resistant to the inducing agent used (Ackermann and DuBow, 1987), or their abundance could be too low to detect significant differences by microscopic counts. In this study, lysogeny was estimated by FLC values derived from both minimum and maximum burst sizes obtained from the literature. Although both estimates lead to different FLC values, they do not alter the patterns and conclusions concerning lysogeny, and similar correlations are found with both values (Figure 1). Consequently, we will use the term FLC irrespective between the FLC_{minBS} and FLC_{maxBS} in this discussion.

Induction assays using mitomycin C

This is the first report on the combined use of mitomycin C and fluorescent hybridization within natural bacterioplankton communities, although it has already been successfully applied in the field of cancer research (Surrallés *et al.*, 1995; Arutyunyan *et al.*, 2004). The absence of significant changes in the hybridization efficiency, assessed by the % EUB between the controls and mitomycin C treatments, indicated that this antibiotic did not interfere with the hybridization. However, a major question concerns whether the observed changes in BCC were only due to the induction of the lysogens, or whether the antibiotic effect of mitomycin C also had a role. In the eight systems in which induction was not detected, the comparison of the control and the treatment samples allowed us to identify the specific antibiotic effect of mitomycin C on the BCC. Among the five selected phylogenetic bacterial groups, this effect on the Δ_P values was very low, albeit variable (Figure 2a). As a result, the antibiotic effect alone did not significantly alter the dominance patterns, as illustrated in Figures 3a and b. Thus, in this study, we consider that the antibiotic effect of mitomycin C can be neglected, whereas assuming that specific group growth and grazing rates were not significantly affected by mitomycin C additions, and that the clear changes in BCC faithfully reflect the induction of lysogens.

Changes in BCC upon induction and ecological considerations

Interactions between virulent viruses and bacteria are a major driving force shaping the BCC in aquatic systems (Winter *et al.*, 2010). Whether lysogeny also influences bacterial diversity is still a matter of debate, but the abundance of lysogens in aquatic systems makes them potential key players (Jiang and Paul, 1996; Casjens and Hendrix, 2005). Using a quantitative approach, we show that induction has a strong effect on the BCC, changing the dominance patterns in both types of ecosystems (Figures 3c and d). These significant changes of the BCC were statistically decomposed by the variance partitioning analysis, showing that FLC was the main explanatory factor for the Δ_P values.

Our results show that in both types of ecosystems, inducible lysogens are not distributed evenly through the targeted phylogenetic groups, and further reveal a consistent pattern in which the most abundant phylogenetic groups contain the highest proportions of inducible lysogens (Table 2, Figures 2b and c). For example, the Bacteroidetes, abundant in both ecosystems, showed important

negative Δ_P values of –5.7 and –16.1% in the reservoirs and lagoons, respectively. In the lagoons, the α-proteobacteria, second most abundant group, was the one with the most negative Δ_P of –8.1%; and in reservoirs, the β-proteobacteria, the most abundant group, had the most negative Δ_P of –8.7%, albeit a large range of values. Such results are in agreement with previous findings indicating that certain bacterial groups contain significant proportions of lysogens or prophage elements (Hewson and Fuhrman, 2007; Paul, 2008). In the case of the Bacteroidetes, Salcher *et al*. (2007) have previously argued that this group could be more sensitive to viral infection than other groups, and we further suggest that this infection could be of a lysogenic origin. In contrast, the averaged Δ_P values of the least abundant groups measured in these systems were often close to zero. It thus appears that there is a connection between the relative abundance of the major phylogenetic groups and their susceptibility to establish lysogeny.

We also studied the impact of induction on the α-proteobacteria SAR11 clade, which comprises species considered as the most successful microorganisms on earth (Morris *et al*., 2002). Members of the SAR11 clade were well represented in lagoons (10% on average) and minor in reservoirs (less than 0.5%), but were virtually not induced in both systems. This might be an indication that lysogeny is not widespread within this clade, consistent with the finding that only few representatives of this group contain inducible prophages (Hewson and Fuhrman, 2007). It is, therefore, probable that other clades within the α-proteobacteria, such as the Roseobacter, abundant in productive ecosystems (Alonso-Saez *et al*., 2007), comprise more lysogens than SAR11, and strongly contribute to the global Δ_P of the α-proteobacteria. Thus, the contrasting Δ_P values between SAR11 and α-proteobacteria further reveal that lysogeny may also be unevenly distributed among the members of a major phylogenetic group.

Although the investigated phylogenetic groups covered between 72 and 90% of the Bacteria, the observed patterns only refer to 43 (reservoirs) and 69% (lagoons) of the *in situ* total prokaryotic assemblage. The missing fraction might be explained by other groups inhabiting these ecosystems, such as Archaea and Actinobacteria (Amann and Fuchs, 2008), which were not investigated here. This demonstrates the need to target a larger number of phylogenetic groups to refine the observed links between the different phylogenetic groups and their susceptibility to establish lysogeny.

Links between lysogeny and host metabolic features

Extensive literature from phage–host systems and field studies suggest that lysogeny is favored when hosts' growth rates may not sustain a fast and important viral replication (Gottesman and Oppenheim, 1994; Weinbauer, 2004; Long *et al*., 2008 and references therein). Our results are in agreement with such hypotheses. Indeed, the proportion of lysogenic cells decreased with increasing bacterial community activity, determined by two different proxies (Figure 1). As both proxies did not explain FLC in both types of ecosystems, it highlights the possibility that the metabolic nature of the signal for the 'lysogenic decision' may differ between systems. This could be attributed to differences in BCC, and to specific intracellular composition, precluding the application of a single model.

One may question whether the relationship between bacterial activity and lysogeny at the community level also holds true when the Δ_P and the specific activity of each phylogenetic group are considered. Our results show that the α- and β-proteobacteria with lower activity in coastal lagoons and in reservoirs, respectively, were more induced (Figure 4). Within these phylogenetic groups, the occurrence of lysogeny is thus dependent on their metabolic activity, in a very similar way to the one observed for the entire community (as depicted in Figure 1). Furthermore, the multiple regression analysis confirms that the bacterial community metabolism significantly influences the observed changes in the Δ_P of the phylogenetic groups. This link was not observed for the other lysogen-containing groups, such as the Bacteroidetes or the γ-proteobacteria. The cellular characteristics favoring lysogeny in these phylogenetic groups remain unclear. We envisage two hypotheses for the absence of link between the bacterial metabolism and lysogeny, which are not mutually exclusive: (i) certain bacterial species may only allow a lysogenic replication pathway. For example, most pathogenic bacteria are lysogens (Brüssow *et al*., 2004). But the existence of exclusively lysogenic hosts in aquatic systems remains to be demonstrated. (ii) The intrinsic low activity of a bacterial group may only allow lysogeny. The Bacteroidetes have been repeatedly found to grow more slowly than other phylogenetic groups (Fuchs *et al*., 2000; Simek *et al*., 2001; Zubkov *et al*., 2001, this study). This metabolic feature may thus represent a signal for the establishment of lysogeny.

Lysogeny as a structural force of bacterial communities

To date, lysogeny has not been considered within the conceptual frame of the virus-mediated control of bacterial community diversity. Lysogeny is characterized by two distinct phases that could influence the BCC with contrasting outcomes. The establishment of lysogeny confers immunity against infection by the same or closely related phages, and prophages can also change the growth properties of their host (Levin and Lenski, 1983; Marsh and Wellington, 1994), generally leading to the increase in their abundance (Edlin *et al*., 1975; Paul, 2008). Here, we propose that these benefits of lysogeny also exist within natural bacterioplankton, probably

conferring a selective advantage to some groups that will eventually become abundant in the community. Thus, by favoring some taxa *in situ*, lysogeny can decrease the community evenness.

On the other hand, induction of the prophage is lethal for the cell. Hence, lysogeny also confers vulnerability towards the environmental conditions that favor induction. This effect is particularly pronounced if there is a higher prevalence of lysogeny among the abundant phylogenetic groups, as observed in this study. Previous studies with natural communities or phage–host systems highlighted the importance of the *in situ* bacterial group abundance for the type of viral infection (Middelboe *et al.*, 2001; Bouvier and del Giorgio, 2007; Hewson and Fuhrman, 2007), in which it was postulated that the least abundant cells could be more susceptible to lytic infection, and that dominant cells might acquire some level of resistance. In the light of our results, we propose that rare phylogenetic groups are less sensitive to lysogenic infections, whereas the most abundant groups are more sensitive to lysogenic infections.

Finally, it also appears that in some cases, minority groups, for example, β-proteobacteria and SAR 11 were able to take advantage from the lysis of lysogens (that is, positive Δ_P values), most likely benefiting from lysis products, such as proteins and amino acids (Cottrell and Kirchman, 2000; Tripp *et al.*, 2008), or from the release of competition with the major phylogenetic groups (Salcher *et al.*, 2007). On the long run, induction may regulate diversity, allowing the less abundant cells to coexist and leading to an increase in species evenness. This increase is also the expected consequence of a ‘killing the winner’ regulation (Winter *et al.*, 2010). Although both effects can have contrary results on the evenness of BCC, there is no data available yet on the dynamics and ecological significance of such virus-mediated processes. Overall, we show that in addition to lytic infection, lysogeny is a driving force for structuring bacterioplankton assemblages both in freshwater reservoirs and in coastal lagoons.

On the basis of the experimental results from two types of ecosystems, we propose a scenario in which inducible lysogens are not evenly distributed in all major phylogenetic groups, and in which the dominant groups contain more inducible lysogens, both in the freshwater and coastal lagoon ecosystems. This pattern was due to host phylogenetic identity or lower activity, both being not mutually exclusive. We further propose that both the establishment of lysogeny and induction events may result in strong changes in the structure of bacterioplankton communities, arguing that lysogeny can function as a significant structural force of bacterial communities. Whether these experimental evidences and patterns remain when multiple inducing agents are applied remains to be investigated.

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