Science of the Total Environment 493 (2014) 872–882

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: A practical approach for a critical review

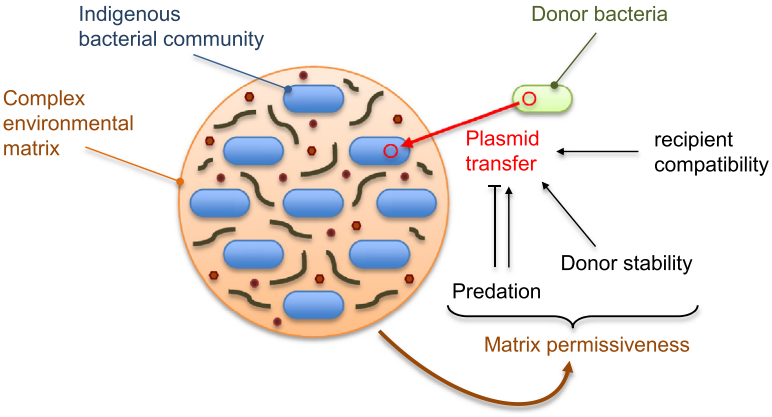
Xavier Bellanger, Hélène Guilloteau, Sébastien Bonot [1], Christophe Merlin *

Université de Lorraine and CNRS, LCPME, UMR 7564, 15 Avenue du Charmois, F-54500 Vandoeuvre-lès-Nancy, France

HIGHLIGHTS

- We examined three methods for assessing plasmid transfer in environmental matrices.
- Isolation of transconjugants by culture is impaired by background antibioresistances.
- Detection of plasmid transfer by fluorescence is unsuitable for environmental samples.
- Molecular-based methods allow detecting rare successful plasmid transfer events.
- Eukaryotic predation both promotes and limits plasmid transfer in natural communities.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 30 April 2014
Received in revised form 16 June 2014
Accepted 17 June 2014
Available online xxxx

Editor: D. Barcelo

Keywords:
Plasmid transfer
pB10
Antibiotic resistance dissemination
Environmental bacterial communities
Eukaryotic predation

ABSTRACT

Plasmid-based dissemination of antibiotic resistance genes in environmental microbial communities is a matter of concern for public health, but it remains difficult to study for methodological reasons. In this study, we used the broad host range plasmid pB10 to compare and to point out the main drawbacks of the three different approaches currently used to evaluate plasmid transfer in natural communities. Culture-based selection of transconjugants appeared to be compromised by high prevalence of antibiotic resistances among natural communities, unless high loads of initial pB10-donor inocula were used. Fluorescence-based detection of transconjugants reached a dead-end consequently to the narrow host range of bacteria expressing fluorescent proteins from a genetically modified pB10 plasmid, in addition to the relatively high background level of fluorescence exhibited by some environmental matrices. The molecular-based approach was the only one to provide a mean to detect rare plasmid transfer events following a low but realistic initial pB10-donor inoculation. Whatever the method, culture-based or molecular-based, the detection of successful transfer events in a given environmental matrix seemed to be linked to the initial stability of the donor inoculum. Depending on the matrix considered, eukaryotic predation plays a significant role in either limiting or promoting the plasmid transfer events.

1. Introduction

The great promises once fueled by the discovery of antibiotics are now severely altered by our increasing failure to treat commonly curable infectious diseases. Such failure results from the still rising

* Corresponding author. Tel.: +33 3 83 68 22 30; fax: +33 3 83 68 22 33.
E-mail addresses: xavier.bellanger@univ-lorraine.fr (X. Bellanger), helene.guilloteau@univ-lorraine.fr (H. Guilloteau), bonot@lippmann.lu (S. Bonot), christophe.merlin@univ-lorraine.fr (C. Merlin).
[1] Present address: Centre de Recherche Public Gabriel Lippmann, Département EVA, 41 rue du Brill, 4422 Belvaux Luxembourg.

http://dx.doi.org/10.1016/j.scitotenv.2014.06.070
0048-9697/

occurrence of antibiotic resistances in bacteria following the production and the consumption of antibiotics in therapeutics and animal husbandry (Davies, 2007; Davies and Davies, 2010; Aminov, 2010). The loss in therapeutic potential associated with the dissemination of antibiotic resistances among pathogens has reached such a critical point that some reports are already describing a near-return to pre-antibiotic era (Levy and Marshall, 2004; Roberts and Simpson, 2008). The relationship between antibiotic consumption and dissemination of antibiotics was acknowledged long ago and, so far, much emphasis has been given to the "hospital" context where antibiotics could act as selective agent. However, more recently, it has become obvious that (i) antibiotics still exert effects on bacteria at low and environmentally relevant concentrations (Yim et al., 2006), and (ii) hot spots of antibiotic resistance gene transfer are likely to be found in various environments able to sustain high cell density (Dröge et al., 1999, 2000; Molin and Tolker-Nielsen, 2003; Aminov, 2011; Rizzo et al., 2013).

To date, most of our knowledge regarding the dissemination of antibiotic resistances in the environment relies on retrospective evidences: (i) the increasing occurrences of antibiotic resistances in various receiving environments (Knapp et al., 2010), (ii) the absence of congruence between the phylogeny of the transferred genes and the phylogeny of the corresponding hosts (Sørensen et al., 2005; Lal et al., 2008), (iii) the loss of synteny between closely related genomes resulting from the insertion of acquired DNA in the host genome (Dobrindt et al., 2004), and finally (iv) the association of the acquired resistance genes with mobile genetic elements (MGEs) such as plasmids, phages, transposons and integrons (Partridge, 2011). Despite being obvious when analyzing acquired DNA sequences from bacterial isolates, the transfer of antibiotic resistance genes in complex environments remains difficult to demonstrate when it comes to work with natural bacterial communities. Yet, demonstrating directly the effectiveness of gene transfer in natural communities is a prerequisite to precisely identify environmental hot spots of antibiotic resistance gene dissemination, as well as environmental parameters driving the evolution of bacteria towards more resistant microorganisms.

It is now well understood that antibiotic resistant genes can spread in microbial communities thanks to genetic structures promoting their mobility (Partridge, 2011). So far, most of our experiences have relied on the transfer of conjugative elements (e.g. plasmids) that are believed to play a predominant role in the adaptation of bacteria to antibiotics pressure (Sørensen et al., 2005; van Elsas and Bailey, 2002; Thomas, 2000; Bennett, 2008). To date, three kinds of approaches have been used to monitor the transfer of known conjugative elements, mainly plasmids, in environmental matrices: culture-based, fluorescence-based and molecular-based approaches (Rizzo et al., 2013). Culture-based methods consist in using selective media to enumerate transconjugants after introduction of donor bacteria in environmental samples (Sørensen et al., 2004; van Elsas et al., 2004). These approaches have been used for more than three decades, allowing the identification of several factors affecting plasmid transfer efficiency. Nevertheless, they remain quite limited when indigenous bacterial populations are used as recipients since less than 1% of environmental bacteria are believed to be culturable, and only a fraction of them are able to express the selective marker transferred (Sørensen et al., 2005). Later on, alternative methods making use of plasmids genetically modified with fluorescent protein genes have been developed for the fluorescent detection of transconjugants in complex environments (Sørensen et al., 2005; Geisenberger et al., 1999). These fluorescence approaches advantageously provide information regarding the localization where the transfers take place in complex community structures (Geisenberger et al., 1999; Musovic et al., 2010; Dahlberg et al., 1998), but they are constrained by the need to genetically modify the element studied. More recently, several teams, including ours, have simultaneously developed a molecular biology-based approach, which makes use of quantitative PCR (qPCR) to monitor the dissemination of a given plasmid in the vastness of microbial community DNAs (Bonot and Merlin, 2010; Merlin et al., 2011; Haug et al., 2010, 2011; Wan et al., 2011). Practically, it consists in inoculating microcosms of environmental samples with a donor bacterium and monitoring the evolution of both the plasmid and the initial host DNAs over time. Because conjugative transfer is an intercellular form of DNA replication, the plasmid to donor DNA ratio increases when the plasmid disseminates by conjugation into the indigenous population. This method provides the advantage of considering gene transfer on a wide range of possible indigenous recipient bacteria, culturable or not, but it requires the design of very specific sets of primers and probes for the non-ambiguous quantification of donor and plasmid.

All these methods have been used with various levels of success to pinpoint particular environments or parameters for their propensity to support the transfer of MGEs. Still, as shown here, demonstrating the transfer of MGEs in environmental matrices is often a difficult and tedious task, with no guarantee of success when it comes to work with complex environments hosting already installed microbial communities. In this report, we used the broad host range plasmid pB10, which is highly efficient for conjugative transfer, to revisit each of these methodologies, and to highlight major drawbacks associated with working with complex environmental matrices. Plasmid transfer was studied using environmental microbial communities maintained in microcosms as recipients. Special attention was given to the initial stability of the donor bacteria and the repercussions it could have on the detection of plasmid transfer events. The influence of eukaryotic predation on the persistence of the donor bacteria, and then on plasmid transfer, was explored.

2. Materials and methods

2.1. Bacterial strains and media

Bacterial strains used in the present study are described in Table 1. Bacteria were routinely cultured at 30 °C under agitation (160 rpm) in LB medium (LB Broth Miller, DifcoTM) supplemented with the appropriate antibiotic for selection. Unless stated otherwise, antibiotics were used for selection at the following concentrations: Amoxicillin (Amx): 30 $\mu g.mL^{-1}$; Chloramphenicol (Chl): 20 $\mu g.mL^{-1}$; Kanamycin (Kan): 100 $\mu g.mL^{-1}$; Nalidixic acid (Nal): 20 $\mu g.mL^{-1}$; Rifampicin (Rif): 100 $\mu g.mL^{-1}$; Streptomycin (Str): 10 $\mu g.mL^{-1}$; Sulfamethoxazole (Sul): 150 $\mu g.mL^{-1}$; Tetracycline (Tet): 10 $\mu g.mL^{-1}$. The concentrations of antibiotics were empirically adjusted on *E. coli* for being selective when the genetic determinant for resistance is present (normal growth) and counter selective when it is absent (no growth). For solid media, agar was added at 15 $g.L^{-1}$.

2.2. Construction of gfp-tagged pB10 plasmid derivatives

Plasmid pB10 was mutagenized by inserting a mini-Tn*5*-*gfp* transposon using a procedure close to the one reported by Christensen et al. (1998). An insertion library of mini-Tn*5*-*gfp*, conferring resistance to kanamycin, was obtained in *Cupriavidus metallidurans* CM124 (AE815(pB10)) using a triparental mating with two other *Escherichia coli* strains, the donor JB120 and the helper HB101(pRK600). Insertion clones were recovered on LB Rif (100 $\mu g.mL^{-1}$) Kan (1 $mg.mL^{-1}$), and pooled in 10 mM $MgSO_4$. The pool was further used as donor in a biparental mating with the recipient strain *E. coli* CM125. Transconjugants displaying a pB10 plasmid enlarged by a copy of mini-Tn*5*-*gfp* (i.e. pB10::mini-Tn*5*-*gfp*) were selected on LB Nal (20 $\mu g.mL^{-1}$) Kan (100 $\mu g.mL^{-1}$), and were further checked for the antibiotic resistances normally encoded by pB10. One of these transconjugants (strain CM192), for which plasmid restriction analyses revealed a mini-Tn*5*-*gfp* insertion in the vicinity of pB10 *oriV (supplementary material)*, was retained for this study. Plasmid pB10::mini-Tn*5*-*gfp* was further introduced into various *Proteobacteria* species with a biparental mating between the donor strain *E. coli* CM125(pB10::mini-Tn*5*-*gfp*) and a set of