Science of the Total Environment 548–549 (2016) 131–138

Contents lists available at ScienceDirect

Science of the Total Environment

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

An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance

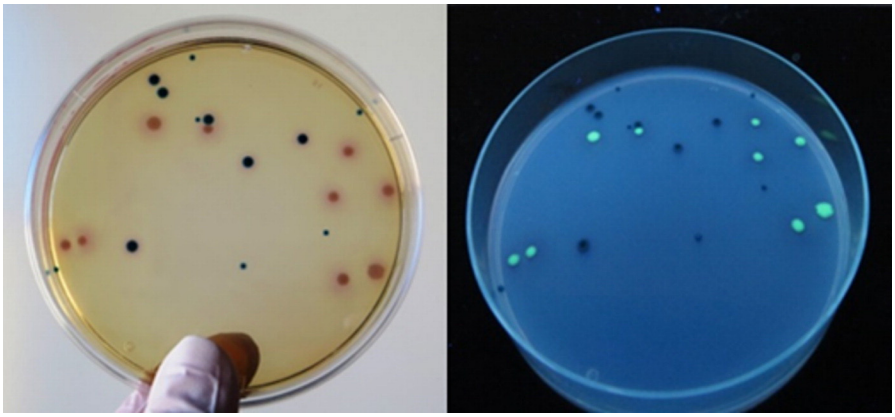
Jekaterina Jutkina, Carolin Rutgersson, Carl-Fredrik Flach, D.G. Joakim Larsson *

Department of Infectious Diseases, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Guldhedsgatan 10, SE-413 46 Gothenburg, Sweden

HIGHLIGHTS

- Minimal concentrations of antibiotics driving transfer of resistance are not known.
- Previous assays do not separate effects on increased transfer from clonal growth.
- Choice of selective agent, medium and reduced conjugation time improved the assay.
- 10 μg/L of tetracycline stimulated horizontal transfer of antibiotic resistance.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 3 November 2015
Received in revised form 8 January 2016
Accepted 8 January 2016
Available online xxxx

Editor: Kevin V. Thomas

Keywords:
Conjugation
Tetracycline
Antibiotic resistance
Mobile genetic elements
Sub-inhibitory concentration

ABSTRACT

Ability to understand the factors driving horizontal transfer of antibiotic resistance from unknown, harmless bacteria to pathogens is crucial in order to tackle the growing resistance problem. However, current methods to measure effects of stressors on horizontal gene transfer have limitations and often fall short, as the estimated endpoints can be a mix of both the number of transfer events and clonal growth of transconjugants. Our aim was therefore to achieve a proper strategy for assessing the minimal concentration of a stressor (exemplified by tetracycline) that drives horizontal transfer of antibiotic resistance from a complex community to a model pathogen. Conditions were optimized to improve a culture-based approach using the bacterial community of treated sewage effluent as donor, and fluorescent, traceable *Escherichia coli* as recipient. Reduced level of background resistance, differentiation of isolates as well as decreased risk for measuring effects of selection were achieved through the use of chromogenic medium, optimization of conjugation time as well as applying a different antibiotic for isolation of transconjugants than the one tested for its ability to drive transfer. Using this assay, we showed that a very low concentration of tetracycline, 10 μg/L i.e. 150 times below the minimal inhibitory concentration of the recipient, promoted horizontal transfer of multiple antibiotic-resistance determinants. Higher concentrations favoured selection of a tetracycline-resistance phenotype along with a decline in the number of detectable transfer events. The described method can be used to evaluate different environmental conditions and factors that trigger horizontal dissemination of mobile resistance elements, eventually resulting in the formation of drug-resistant pathogens.

* Corresponding author.
E-mail address: joakim.larsson@fysiologi.gu.se (D.G. Joakim Larsson).

http://dx.doi.org/10.1016/j.scitotenv.2016.01.044
0048-9697/

1. Introduction

The spread of antibiotic resistance is frequently associated with the high adaptive capacity of microorganisms; bacteria are prone to genetic changes and acquisition of mobile genetic elements by means of horizontal gene transfer (HGT). Rapid acquisition of antibiotic resistance by pathogenic bacteria may occur via cell-to-cell contacts with other bacteria, which carry resistance genes on mobile genetic elements (MGEs) (Bennett, 2008; Huddleston, 2014). This mechanism, known as conjugative transfer, is commonly orchestrated by plasmids, serving as powerful instruments for bacterial evolution and adaptability (Smalla et al., 2015; Thomas and Nielsen, 2005). Although numerous applications of genomic and metagenomic analyses provide evidence of the extensive gene exchange ongoing in nature, exploring the circumstances that trigger HGT as well as learning to detect and monitor these events directly in complex bacterial communities is desirable in order to understand the true extent of this phenomenon and the impact of human activities, including pollution (Aminov, 2011; Heuer and Smalla, 2012).

Recent findings suggest that the largest extent of horizontal transfer of resistance plasmids in the environment occurs in the presence of stressors such as antibiotics and possibly some nanomaterials at very low, sublethal concentrations (Kim et al., 2014; Qiu et al., 2012). Also some metals are found to facilitate transfer of antibiotic resistance (Suzuki et al., 2012). Antibiotic concentrations well below therapeutic levels may serve as signalling molecules (Gillings, 2013) and regulatory substances which induce conjugative transfer, mutagenesis and SOS response, causing specific transcriptional changes as well as provide selective advantage to resistant bacterial strains down to more than 100-fold below minimum inhibitory concentration (MIC) (Andersson and Hughes, 2014). Experiments on HGT in the gastrointestinal tract of gnotobiotic rats indicate that exposure of the gut flora to antibiotics provides enhanced opportunities for conjugative transfer (Bahl et al., 2004; Feld et al., 2008). In the external environment, antibiotic residues are frequently found in human-impacted areas. Concentrations measured in surface water are often in the ng/L range, in sewage treatment plants (STPs) concentrations may extend up to μg/L, and in the vicinity of manufacturing sites even mg/L concentrations can be found (Kümmerer, 2009; Larsson, 2014). The latter represents exposure levels that exceed the MIC of a vast number of bacterial strains of different taxa. Taken together, there is a range of environments and situations where HGT may be induced by subinhibitory concentrations of antibiotics. However, without knowing which concentrations are required to trigger HGT it is difficult both to assess and manage risks.

Assessing the concentration of stressors that promote HGT in complex bacterial communities is challenging since methods proposed so far suffer from a number of weaknesses. Three main approaches, culture-based, fluorescence-based and molecular-based, as well as combinations thereof, have been used to monitor plasmid transfer. In most cases, the donor is a strain carrying a defined mobile genetic element (Bellanger et al., 2014; Bonot and Merlin, 2010; Klümper et al., 2014; Klümper et al., 2015; Rizzo et al., 2013). However, for the purpose of identifying the minimal concentration of a stressor that drives horizontal transfer of resistance, an assay based on a traceable recipient rather than a specific plasmid-bearing donor would have at least two important advantages: (i) the basic principle would better reflect the risk scenario of a complex environmental community as a source for mobile antibiotic-resistance elements to primary and/or opportunistic human pathogens and (ii) the effect on a large variety of potential donors is investigated, each of which may have different sensitivity to the stressor. Indeed, the use of a *gfp*-tagged, traceable recipient with a complex donor community has shown its value in identifying and characterizing conjugative resistance elements in various environments (Binh et al., 2008; Flach et al., 2015; Heuer et al., 2012). However, traditional culture-dependent assays are not optimal for HGT assessment, as possible clonal expansion after conjugation distorts the estimate of actual transfer events (Sørensen et al., 2005). Furthermore, culture-based methods using complex bacterial communities are often problematic due to indigenous multi-resistant bacteria and an ambiguous identification procedure of transconjugants (Bellanger et al., 2014). Thus, despite a variety of known techniques to study HGT, there is a clear need to improve assays to enable determination of the minimal concentrations of chemical stressors that can drive horizontal transfer of resistance determinants in complex bacterial communities.

Our primary aim was therefore to adapt an existing culture-based approach of exogenous isolation of mobile genetic elements to allow the assessment of the minimal concentration of a stressor that drives horizontal transfer of antibiotic resistance from bacteria within a complex bacterial community to a model pathogen. We used the bacterial community of STP effluent as the donor of indigenous mobile antibiotic resistance elements and a *gfp*-marked *Escherichia coli* (Heuer et al., 2002) as the recipient. Transconjugant isolation and identification procedure was improved by using appropriate differential and selective conditions. The reduction of background resistance and clonal expansion related interference was achieved through minimization of conjugation time and use of different compounds for selection and induction. As a proof of concept, we applied this strategy to identify the lowest concentration of tetracycline that would trigger horizontal transfer of resistance. Tetracycline is of profound interest since it represents a family of broad-spectrum antibiotics commonly used to treat both humans and animals. It is frequently detected in the environment at ng/L up to low μg/L concentrations (Daghrir and Drogui, 2013). Hence, serious concerns have been raised about the possible role of subtherapeutic concentrations of tetracycline in regard to the promotion of antibiotic resistance.

2. Materials and methods

2.1. Bacterial source and culture preparation for HGT assays

E. coli was chosen as recipient for HGT monitoring since it is a common cause of bacterial infections (Kaper et al., 2004) and a typical host for a broad spectrum of plasmids known to a carry a wide diversity of antibiotic resistance genes (Carattoli, 2009). Importantly, the *E. coli* strain CV601 used here can be distinguished from indigenous environmental *E. coli* on the basis of kanamycin and rifampicin resistance markers and *gfp* expression when exposed to UV light (Heuer et al., 2002). The determined MICs of tetracycline and sulfamethoxazole for the strain CV601 are 1.5 mg/L and 16 mg/L respectively (Flach et al., 2015).

The recipient was cultured in LB broth in presence of kanamycin (50 mg/L) at 30 °C shaking overnight after which it was diluted 1/20 in fresh LB broth and grown for a few hours while preparing the donor community. The cells were washed twice in PBS to remove the traces of antibiotics and re-suspended in a small volume of PBS. The OD_{600nm} of the culture was adjusted to around 0.7.

In order to provide a diverse source of mobile resistance elements and donors, treated effluent from Scandinavia's largest STP (Ryaverket in Gothenburg, Sweden) served as a complex donor community. All experiments were performed within 24 h of effluent collection. Samples were kept at 4 °C until analysis. Effluent was filtered through 0.45 μm pore size S-Pak filters (Millipore Corporation, Bedford, USA) after which the filters were cut into small pieces and placed into tubes containing 25 mL of PBS and sterile glass beads. The tubes were vortexed for 10 min to facilitate detachment of bacteria from filters. The donor suspensions were decanted to new tubes and centrifuged for 15 min at 2755 × g. The pellets were washed with PBS twice and re-suspended in PBS to a final volume which corresponded to OD_{600nm} of 1.3–1.5.