Institut Pasteur
Research in Microbiology 163 (2012) 200–210
www.elsevier.com/locate/resmic

Fate of invading bacteria in soil and survival of transformants after simulated uptake of transgenes, as evaluated by a model system based on lindane degradation

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Received 25 August 2011; accepted 19 January 2012
Available online 10 February 2012

Abstract

Emergence of bacteria carrying new traits resulting from mutations, gene synthesis by gene-shuffling or acquisition of exogenous DNA underpins the need to better understand factors influencing their spread and establishment. Studies of soils may be difficult, since the gene of interest is often already present in high numbers. The gene *linA*, responsible for the first dechlorination steps during degradation of lindane (γ-hexachlorocyclohexane), has low background levels in soil and is simple to detect. Development of transgenic plants containing *linA* and newly proposed approaches to bioremediation by in situ electrotransformation after addition of a vector carrying this gene call for documentation on the fate of bacteria that incorporate it. We inserted *linA* into the broad-host-range conjugative RP4-plasmid and transferred it to different soil bacteria which were inoculated into soil microcosms in the presence/absence of lindane. Similar experiments were performed using *Sphingobium francense* Sp+, which carries all genes for complete lindane degradation. This strain increased in numbers during lindane mineralization, but other bacteria increased more, resulting in a modified bacterial community structure. The engineered strains decreased below the detection limit, but rose in numbers after nutrient addition, demonstrating that new invading bacteria may persist in soil in the form of small populations over extended time periods.

Keywords: Lindane; Transgene; Bacterial community; Indigenous bacteria

1. Introduction

The huge bacterial diversity of many environments, especially soil, raises questions concerning how such diversity developed and how it is maintained. Recent estimates show that soils may harbor from 10,000 to more than one million different bacterial genome types per g (Gans et al., 2005; Roesch et al., 2007; Torsvik et al., 2002). However, most of this biodiversity is composed of the rare biosphere consisting of bacterial populations with a limited number of cells. Bacterial communities, at least in unsaturated soils, are therefore characterized by substantial evenness (Elshahed et al., 2008; Gans et al., 2005; Huse et al., 2010; Zhou et al., 2002). Thus, forces appear to be involved that prevent most populations from becoming dominant. The reasons are poorly understood, and despite many research efforts, it is still unclear whether, and under which conditions, bacteria that spread to a new environment are able to establish themselves as new dominant populations. More knowledge as to why, when exposed to a new environment, some bacteria are successful and others are not is crucial for improving our understanding of bacterial evolution and adaptation. Such

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doi:10.1016/j.resmic.2012.01.007

knowledge is also needed for a number of related issues such as evaluation of risks or benefits connected with accidental or deliberate release of microorganisms selected or genetically engineered for medical and environmental purposes.

Studies of the spread and establishment of new foreign genes or bacteria in soil are complicated by the wide genetic diversity of the indigenous microbial community. To circumvent such problems, the gene that is studied for its fate as well as its bacterial host should ideally not have been previously present in the soil microbial community, and it should provide specific physiological traits to its host. Thus, with low background interference, it should be easily detected using a simple method. Although genes with such characteristics are rare in soil bacteria, they can be detected in some bacteria that have recently acquired the capacity to degrade xenobiotic compounds. This is apparently the case with the gene *linA* that encodes a dechlorinase enzyme involved in the two first dechlorination steps of the multichlorinated organic compound lindane (1,2,3,4,5,6-c-hexachlorocyclohexane, or γ-HCH) (Cérémonie et al., 2006; Nagata et al., 1993). This multichlorinated organic compound has been extensively used worldwide as an insecticide over the past several decades. It is a bio-accumulative substance which acts as a neurotoxin and possible human carcinogen (Guan et al., 1995), and has therefore been banned in most countries for use in agriculture. It is persistent in the environment and high levels can still be measured in many areas (Ao et al., 2009; Kashyap et al., 2002; Mukherjee and Gopal, 2002; Nawab et al., 2003; Noren and Meironyte, 2000; Simonich and Hites, 1995). Although several other chromosome- or plasmid-borne genes (Kumari et al., 2002) are directly involved in complete degradation of lindane to CO_2, it is the first two dechlorination steps that appear to be crucial. Only a few strains of bacteria capable of degrading lindane aerobically have been isolated, and only from strongly contaminated soils. Several of these belong to the genera *Sphingomonas*/*Sphingobium* (Boltner et al., 2005; Cérémonie et al., 2006; Manickam et al., 2008; Mohn et al., 2006; Nagata et al., 2005; Yamamoto et al., 2009), although other genera are also represented (Gupta et al., 2000; Nawab et al., 2003; Pesce and Wunderlin, 2004). In non-contaminated soils, such lindane-degrading bacteria can generally not be detected and PCR fails to amplify the *linA* gene (Thomas et al., 1996). These findings and the fact that the *linA* gene may be a novel gene resulting from natural shuffling of various exogenous DNA fragments (Boubakri et al., 2006) confirm its interest as a model for investigating the fate of new genes and new bacterial populations in soil environments. In addition, transgenic plants containing a *linA* gene from *Sphingomonas paucimobilis* have recently been patented (De Lorenzo Prieto and Gonzalez Pastor, 2007). It is possible that naturally transformable bacteria in the soil could develop genetic competence and acquire DNA from such plants (Kay et al., 2002a, 2002b; Pontiroli et al., 2009). Furthermore, a new approach to in situ bioremediation was proposed by Lyon and collaborators (Lyon et al., 2010) wherein the *linA* gene was inserted into a broad-host-range plasmid which was added to soil. After treating soil with a pulsed electric field, the authors showed increased degradation of lindane, which was explained by uptake of the *linA*-containing plasmid by soil bacteria through electrotransformation. We see two possible explanations for this; either the bacteria that took up the plasmid already contained other genes needed for degradation of lindane, or the plasmid was taken up by bacteria having no other lindane degradation genes, but that performed the first dechlorination steps while other bacteria in their surroundings continued degradation of the metabolites. The present study was designed to determine whether degradation of lindane took place in soils where the *linA* gene was taken up by bacteria not carrying other lindane degradation genes, and to investigate the fate of such host bacteria. We performed a series of experiments in which a *lin*A-carrying plasmid was introduced into three different common soil bacteria which lacked the other genes for complete degradation of lindane. These *linA*-containing isolates were inoculated into soil microcosms containing different levels of lindane. Bacteria were quantified by bacterial counts on lindane-containing medium, and degradation of lindane was measured using a chloride ion selective electrode. Effects on total bacterial community structure were evaluated using PCR-DGGE and phospholipid fatty acid (PLFA) fingerprinting. The results were compared to those obtained when *Sphingomonas francense*, which contains both the gene *linA* and other genes for lindane mineralization, was used as inoculum.

2. Materials and methods

2.1. Bacterial strains and plasmids

The gene *linA*, coding for the first two dechlorination steps in degradation of lindane, was inserted into self-transmissible wide-host-range plasmid RP4 (Thomas and Smith, 1987), together with a *gfp* cassette (Chalfie et al., 1994; Dahlberg et al., 1998; Miller and Lindow, 1997). The resulting plasmid was introduced into three different bacterial strains, *Acinetobacter* sp. BD413, *Alcaligenes faecalis* IS-18R and *Rhizobium radiobacter* NCCB2549. These strains were chosen because they represent common soil bacterial species; moreover, they all grow at pH below 5.2–5.3 (*A. faecalis* and *Acinetobacter* sp. BD413) or at pH below 5.5 (*R. radiobacter*) and were therefore expected to tolerate the local pH decrease caused by lindane dechlorination. These transconjugants were inoculated into soil microcosms containing different levels of lindane. The fate of these strains was compared with that of *S. francense* strain Sp+ (Pal et al., 2005; Thomas et al., 1996), which carries all genes necessary for complete degradation of lindane (Cérémonie et al., 2006).

Construction of the RP4 derivative was performed as follows: Forward primer linA1 5′-CAAGCTTAGCTCACGA CGCTTTACAAC-3′ and reverse primer linA2 5′-TGGATC-CACTCATCTCCAATGCGAGTC-3′ were designed and used for amplification of the *linA* gene, including its promotor, from *S. francense* strain Sp+ (total fragment length 1.2 kb). The PCR reaction mixture was as follows: (50 μl): 1×PCR buffer, 2.5 mmol l^{-1} $MgCl_2$, 10 pmol primers, 0.2 mmol l^{-1}