Long-term manure exposure increases soil bacterial community potential for plasmid uptake

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Running title: Manure increases community plasmid uptake

Abstract:

Microbial communities derived from soils subject to different agronomic treatments were challenged with three broad host range plasmids, RP4, pIPO2tet, and pRO101, via solid surface filter matings to assess their permissiveness. Approximately 1 in 10,000 soil bacterial cells could receive and maintain the plasmids. The community permissiveness increased up to 100% in communities derived from manured soil. While the plasmid transfer frequency was significantly influenced by both the type of plasmid and the agronomic treatment, the diversity of the transconjugal pools was purely plasmid dependent and was dominated by β- and γ-Proteobacteria.

Text

Rapid adaption of bacterial communities to changing environmental conditions is believed to rely on lateral transfer of mobile genetic elements, such as plasmids, as one indispensable mechanism (Sørensen et al., 2005; Thomas and Nielsen, 2005; Heuer and Smalla, 2012). One of the crucial parameters that determines the extent of conjugal plasmid transfer is community permissiveness, defined as the fraction of a microbial community able to receive a given plasmid (Musovic et al., 2010). In agricultural soils, seasonal application of manure and fertilizers provokes intense and immediate changes to soil physical-chemical conditions that might modulate horizontal gene transfer (HGT). Indeed, both an increased nutrient availability (van Elsas et al., 2003) and the introduction of selective stressors like metals or antibiotics (Newby and Pepper, 2002; Heuer et al., 2011) may lead to increased rates of plasmid transfer. Apart from this immediate stimulation of HGT, it remains unknown whether agronomic soil treatment may have long-term effects on permissiveness. To test this, we investigated permissiveness towards three broad-host-range plasmids (Table 1) in four communities isolated from agricultural soils subjected to different long-term seasonal treatments (Table 2).

Microbial communities from three plots (Untreated, Manured and Nitrate-Phosphate-Potassium-fertilized (NPK)), at the long-term CRUCIAL experimental site (Taastrup, Denmark) (Magid et al., 2006; Poulsen et al., 2013), were sampled (SI2) in order to assess permissiveness. Treatments at the field site were in place for 8 years at the time of sampling. The manure derived from a conventional dairy cow farm. The estimated accumulative applications of C-N-P-K were estimated at 62800-3768-1184-2979 (manured), 0-840-120-400 (NPK) and 0-0-0-0 (untreated) kg/ha respectively (Magid et al., 2006).

As reference, soil from an untreated plot of the well-known Rothamsted Park Grassland (Rothamsted, United Kingdom) (Silvertown et al., 2006) site was included. From the chosen soils, indigenous bacterial communities were isolated by Nycodenz®-extraction (Musovic et al., 2010) and challenged with exogenous plasmids via solid-surface filter matings (Musovic et al., 2010). *Pseudomonas putida* KT2442 (Bagdasarian et al., 1981) served as the plasmid donor strain (Table 1). Conjugation events were detected by epifluorescence microscopy, allowing their quantification (Musovic et al., 2010).

For all tested combinations, community permissiveness ranged from $8.24*10^{-5}$ to $4.56*10^{-4}$ conjugations per recipient (Figure 1). Permissiveness was consistently higher (up to 3 times) for the soil-derived pIPO2tet plasmid than for the other two IncP plasmids, RP4 and pRO101. Therefore, for both sampling sites, the introduced plasmid significantly influenced the permissiveness ($p_{CRUCIAL}<0.001$; $p_{Rothamstead}<0.001$). Additionally, in CRUCIAL soils, a significantly higher permissiveness was measured for the manure treated community compared to the untreated control ($p_{RP4}=0.041$; $p_{pIPO2tet}=0.001$). Previous studies have suggested that manure treatment may result in hot-spots of gene transfer due to increased nutrient availability and cell density (van Elsas et al., 2003) with transfer frequencies increasing by up to one order of magnitude (Götz and Smalla, 1997). However, this does not explain why we observed stimulation of permissiveness by manuring because, in our assay, heterogeneities in nutrient or cell density mating were excluded as matings were carried out under standard nutritional conditions on soil extract medium (Musovic et al., 2010). While raised levels of plasmid shuttled tetracycline resistance genes were shown to occur in soils treated with piggery manure slurry (Agersø et al., 2006) or chicken waste (You et al., 2012) in selective and enriching environments, this study is the first to indicate

72 that long-term manure treatment also changes the community permissiveness towards
73 newly introduced plasmids under neutral conditions.

74 Nutrient addition has previously been shown to enhance gene transfer frequency in soils
75 (Smets et al., 1995; Nielsen and van Elsas, 2001). To study the effect of fertilization, we
76 investigated the permissiveness of RP4 in the NPK-fertilized soil bacterial community.
77 Permissiveness in the NPK-treated soil was similar to the untreated control (p=0.79) and
78 significantly lower than in the manured one (p=0.016). Therefore, higher activity through
79 previous nutrient addition is not the reason for increased permissiveness in manured soil.
80 It thus seems that increased permissiveness was not due to immediate nutrient effects, or
81 increased cell density in hot-spots. However, increased permissiveness might have been
82 intrinsic to the community. Hence, we examined the diversity of the transconjugal pools, to
83 test whether they differ between soil treatments.

84 Random transconjugants were isolated from the matings using micromanipulation
85 (Musovic et al., 2010) to analyze, if apart from increased transfer frequencies, the
86 phylogenetic composition of the transconjugal pool changed due to long-term manure
87 application. Successfully isolated transconjugants were subjected to 16S rRNA
88 sequencing (Musovic et al., 2010). Sequences were analyzed using mothur v.1.30.0
89 (Schloss et al., 2009) and the SILVA database (Quast et al., 2013). The sequences have
90 been submitted to GenBank and can be accessed under number KF590708 - KF591079.

91 Principal Coordinate Analysis (PCoA) revealed transconjugal pools separating primarily by
92 introduced plasmid (Axis 1, Figure 2). Independent of sampling site or treatment, pRO101,
93 RP4 and pIPO2tet were associated with significantly different transconjugal pools as
94 revealed by AMOVA (Excoffier et al., 1992) (p=0.007). Surprisingly, the number of OTUs

was lowest for pIPO2tet (SI Table 1), the plasmid exhibiting the highest permissiveness. Although the effective phylogenetic host range in the tested soils appears smallest for pIPO2tet, it might exhibit higher transfer frequencies, resulting in a higher total permissiveness, possibly due to its nature as cryptic plasmid (van Elsas et al., 1998). High transfer frequencies are especially important for the maintenance of those plasmids not conferring any beneficial traits to their hosts.

The second dimension of PCoA separated all transconjugal pools from the CRUCIAL site from those from Rothamsted ($p_{RP4}<0.001$; $p_{pIPO2tet}<0.001$). This separation likely derives from differences in their original bacterial community composition. Meanwhile, an earlier study – comparing deeply sequenced 16S rRNA community libraries – revealed no major difference in the total bacterial community composition for different treatments at the CRUCIAL site (Poulsen et al., 2013), hinting towards the observed grouping of those transconjugal pools.

While plasmid and sampling site both affected transconjugal pools, no effect of agricultural treatment was detected ($p>0.9$) for any of the plasmids in the CRUCIAL soil. A closer look at the six corresponding phylogenetic profiles (Figure 3) confirms that transconjugal pools of the same plasmid within different soil communities are closely related. For instance, *Enterobacteriacae* sequences can only be found in both transconjugal pools associated with pRO101, although their relative fractions differ. All six pools were dominated by β- and γ-Proteobacteria. α-Proteobacteria, Flavobacteria and Sphingobacteria were detected in lower abundance, revealing a wide variety of transconjugants (SI Table 1), in spite of the low number of isolates. High-throughput analysis of transconjugal pools could in the future lead to new insights in the extent of plasmid transfer in soils.

The similarity of transconjugal pools across soil treatments parallels that of the total bacterial community composition of CRUCIAL soils (Poulsen et al., 2013). Therefore, the increased community permissiveness in manured soil cannot be explained by difference in community diversity. Increased seasonal nutrient availability can also be ruled out, since high permissiveness was not observed for NPK-fertilized soil. Potentially, a higher indigenous plasmid content in the community associated with manure applications (Marti et al., 2013), can result in a higher permissiveness towards additional plasmids, by increasing the mating potential of the plasmid bearing cells. Additionally, the introduction of diverse plasmids through manure applicationmay increase the community's permissiveness. Indeed previous hosting of a plasmid has been shown to significantly increase the permissiveness towards its renewed uptake in a *Dickeya* strain (Heuer et al., 2010).

On the other hand, the periodic introduction of stressors, such as metal ions (Nicholson et al., 1999) or antibiotics (Christian et al., 2003) present in manure, might lead to selection mechanisms favoring more robust populations adapted to environmental changes through increased permissiveness towards foreign DNA uptake (Heuer et al., 2008). These selection mechanisms for more permissive strains do not have to be associated with changes in the phylogenetic profile of the community, since permissiveness towards broad host range plasmids of isolates that are genetically indistinguishable by 16S rRNA analysis from identical field plot can differ by more than 2 orders of magnitude (Heuer et al., 2010). Still, the exact mechanisms of the observed enhanced permissiveness are yet to be elucidated. High-throughput analysis of the transconjugal pools could thus lead to insights into community dynamics of plasmid transfer in soil.

Here, we confirmed that soil communities can serve as significant reservoirs for exogenous conjugal plasmids. Approximately 1 in every 10,000 indigenous cells could receive and maintain the studied plasmids. This frequency is increased up to 100% in soils subject to manure treatment. Such an increase in the permissiveness of soil communities and therefore in their potential for contributing to the spread of antibiotic resistance genes is remarkable, making it crucial to elucidate the underlying mechanisms in further research with higher sampling depth. Additional work investigating dissemination and mobilization capacity of more diverse genetic elements among bacterial communities from differently treated soils is therefore necessary.

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235 carrying plasmid in chicken-waste-impacted farm soil. *Appl. Environ. Microbiol.* **78**:
236 3203–13.

237

238

239 Table 1: Plasmids[a] carried by the red fluorescent donor strain, *Pseudomonas putida*

240 KT2442 *DsRed*::*lacIq*

Plasmid	Inc-group	Type	Phenotype	Host range	References
RP4::P*lac*::*gfp*	IncP-1α	Resistance	Tet^R, Amp^R, Km^R	broad	(Barth and Grinter, 1977)
pIPO2tet::P*lac*::*gfp*	(IncQ-mobilizer)	Cryptic	Tet^R	broad	(Tauch et al., 2002)
pRO101::P*lac*::*gfp*	IncP-1β	Catabolic	Tfd^+, Hg^R, Tet^R,	broad	(Harker et al., 1989)

241 [a]Plasmids are tagged using a TN5 cassette with a constantly expressed *gfp* gene that is

242 *lacI* repressed in the donor strains.

243

244

245 Table 2: Origin of the recipient microbial communities

Soil origin	Soil type	Soil treatment	Coordinates	References
CRUCIAL Taastrup Denmark	agricultural	Untreated	(55.681 N, 12.276 E)	(Poulsen et al., 2013; Magid et al., 2006)
		Manured	(55.681 N, 12.276 E)	(Poulsen et al., 2013; Magid et al., 2006)
		NPK-fertilized	(55.681 N, 12.276 E)	(Poulsen et al., 2013; Magid et al., 2006)
Park Grass Rothamsted, UK	grassland	Untreated	(51.811 N, - 0.377 E)	(Silvertown et al., 2006)

246

247

Figure 1: Transfer frequency of the introduced plasmid to the soil indigenous bacterial community after 48 hours of incubation on filters on soil extract medium. Values are displayed as the mean of filter triplicates with standard deviation.

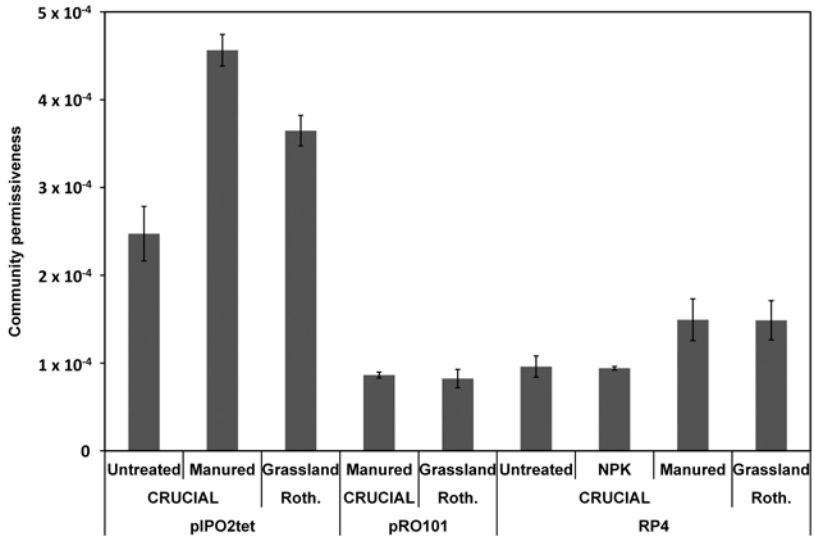


Figure 2: Principal Coordinate Analysis (PCoA) of the sequenced transconjugal pools. Plasmids: pIPO2tet (red), pRO101 (green), RP4 (blue); Soil microbial communities: CRUCIAL Manured (diamond), CRUCIAL Untreated (triangle), CRUCIAL NPK-fertilized (square), Rothamstaed Park Grass (circle)

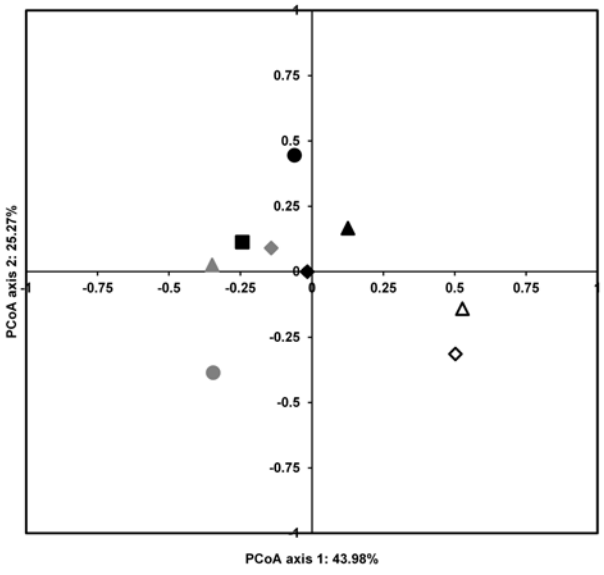
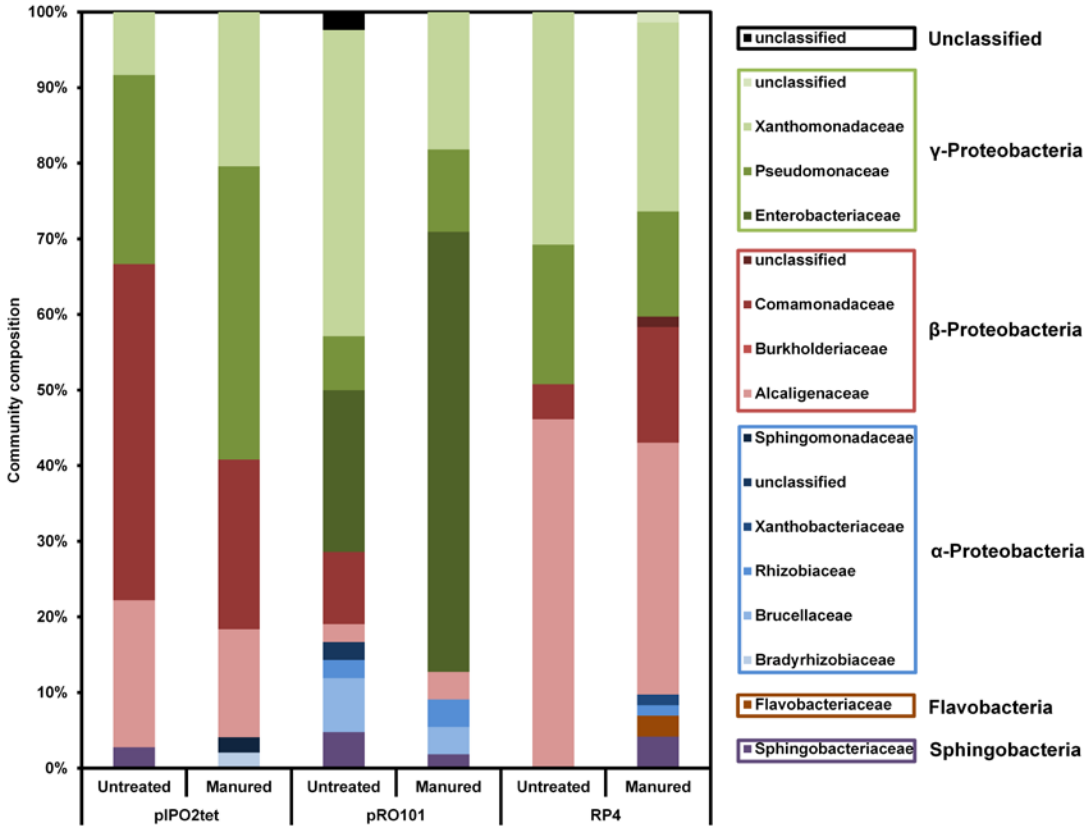


Figure 3: Phylogenetic analysis of the transconjugal pools originating from CRUCIAL Manured and CRUCIAL Untreated soil microbial communities for all 3 tested plasmids.



SI Table 1: Diversity of transconjugants sorted by OTUs and occurrence per transconjugal pool.

OTU	Kingdom	Phylum	Class	Order	Family	Genus	pIPO2tet				pRO101		RP4			total sequences
							CRUCIAL			Rothamstead	CRUCIAL		CRUCIAL		Rothamstead	
							Untreated	NPK	Manured	Park Grass	Untreated	Manured	Untreated	Manured	Park Grass	
1			Flavobacteria	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	0	0	0	0	0	0	0	1	0	**1**
2						unclassified	0	0	0	0	0	0	0	1	0	**1**
3						Pedobacter	0	0	0	0	0	0	0	2	1	**3**
4		Bacteroidetes					1	0	0	0	0	0	0	1	0	**2**
5			Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	0	0	0	0	1	0	0	0	0	**1**
6							0	0	0	0	0	1	0	0	0	**1**
7							0	0	0	0	1	0	0	0	0	**1**
8					Bradyrhizobiaceae	Bosea	0	0	1	0	0	0	0	0	0	**1**
9					Brucellaceae	Ochrobactrum	0	0	0	1	3	0	0	0	0	**4**
10							0	0	0	0	0	2	0	0	0	**2**
11			Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	0	0	0	1	1	2	0	1	0	**5**
12					unclassified	unclassified	0	0	0	0	1	0	0	0	0	**1**
13					Xanthobacteraceae	unclassified	0	0	0	0	0	0	0	1	0	**1**
14				Sphingomonadales	Sphingomonadaceae	Sphingomonas	0	0	1	0	0	0	0	0	0	**1**
15							7	9	7	2	1	2	27	21	4	**80**
16					Alcaligenaceae	Achromobacter	0	0	0	0	0	0	1	1	0	**2**
17							0	0	0	0	0	0	0	1	0	**1**
18			Betaproteobacteria	Burkholderiales			0	0	0	0	0	0	1	0	0	**1**
19						unknown	0	0	0	0	0	0	1	1	1	**3**
20					Burkholderiaceae	unknown	0	0	0	0	0	0	0	0	5	**5**
21					Comamonadaceae	Variovorax	16	0	11	0	4	0	3	11	0	**45**
22					unclassified	unclassified	0	0	0	0	0	0	0	1	0	**1**
23	Bacteria					Pantoea	0	0	0	0	1	0	0	0	0	**1**
24						unclassified	0	0	0	0	8	25	0	0	0	**33**
25				Enterobacteriales	Enterobacteriaceae	unclassified	0	0	0	0	0	2	0	0	0	**2**
26		Proteobacteria				unclassified	0	0	0	0	0	1	0	0	0	**1**
27						unclassified	0	0	0	0	0	1	0	0	0	**1**
28						unknown	0	0	0	0	0	3	0	0	0	**3**
29							8	4	18	1	2	5	11	10	2	**61**
30							0	0	0	0	1	0	0	0	0	**1**
31						Pseudomonas	0	0	0	0	0	1	0	0	0	**1**
32				Pseudomonadales	Pseudomonadaceae		0	0	0	0	0	0	1	0	0	**1**
33			Gammaproteobacteria				0	1	0	0	0	0	0	0	0	**1**
34							0	0	1	0	0	0	0	0	0	**1**
35						unclassified	0	0	0	1	0	0	0	0	0	**1**
36						unclassified	1	0	0	0	0	0	0	0	0	**1**
37							0	3	4	0	1	0	18	7	10	**43**
38							3	4	6	0	6	8	1	11	3	**42**
39						Stenotrophomonas	0	0	0	0	4	0	0	0	0	**4**
40				Xanthomonadales	Xanthomonadaceae		0	0	0	0	0	2	0	0	0	**2**
41							0	0	0	0	1	0	0	0	0	**1**
42							0	0	0	0	0	0	1	0	0	**1**
43						unclassified	0	0	0	0	4	0	0	0	0	**4**
44						unclassified	0	0	0	0	1	0	0	0	0	**1**
45			unclassified	unclassified	unclassified	unclassified	0	0	0	0	0	0	0	1	0	**1**
46		unclassified	unclassified	unclassified	unclassified	unclassified	0	0	0	0	1	0	0	0	0	**1**
						total OTUs	**6**	**5**	**8**	**5**	**18**	**13**	**10**	**16**	**7**	**46**
						total sequences	**36**	**21**	**49**	**6**	**42**	**55**	**65**	**72**	**26**	**372**

SI Table 1: Diversity of transconjugants sorted by OTUs and occurrence per transconjugal pool. Sequences were analyzed using mothur v1.30.0 (Schloss et al., 2009) and the SILVA database (Quast et al., 2013). These sequence data have been submitted to the GenBank database under submission ID 1656830.

SI 2: Sampling procedure and information

Soil samples of three different treatments were taken at the annually manured CRUCIAL (Closing the Rural Urban Nutrient Cycle) agricultural field site (Taastrup, Denmark) (Magid et al., 2006; Poulsen et al., 2013). Soil samples were collected in late fall 2010. Samples of each treatment were taken from three different plots of this treatment. Each plot was sampled for 1 kg of soil at 15 locations. The resulting soil volume was sieved and homogenized to obtain a representative sample. Twenty grams of this homogenized soil samples were used for Nycodenz®-extraction. The fourth sample was taken from the monitored untreated Park Grass field in Rothamsted (Silvertown et al., 2006) (Rothamsted, UK) in the exact same manner.