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ABSTRACT The frequency of transconjugants were compared for the incompatibility (Inc) P-1 and P-7 plasmids pBP136 and pCAR1 under aerobic and anaerobic conditions. Filter mating assays were performed with one donor strain and one recipient strain using different donors of *Pseudomonas* and recipient strains, including *Pseudomonas*, *Pantoea*, and *Buttiauxella*. Under anaerobic condition, frequencies of transconjugants for both plasmids were 10^1-10^3-fold lower than those under aerobic condition regardless of whether aerobically or anaerobically grown donors and recipients were used. To compare the transconjugant ranges under aerobic and anaerobic conditions, conjugation was performed between the donor of pBP136 and recipient bacteria extracted from environmental samples. Several transconjugants were uniquely obtained from each aerobic or anaerobic condition. Our findings indicate that a plasmid can differently spread among bacteria depending on the oxygen concentrations of the environment.

Keywords: Plasmid, conjugation, transconjugant range, aerobe, anaerobe

The conjugation of plasmids, including their genetic cargo, e.g., catabolic genes and antibiotic resistance genes, promotes bacterial evolution and adaptation in natural environments. In-depth studies investigating the conjugation of plasmids have been performed under aerobic conditions, although many (conjugative) plasmids have been found in facultative anaerobes, such as *Escherichia coli*, and obligate anaerobes, including *Clostridium*, *Geobacter*, and *Porphyromonas* (Shintani et al., 2015, Galata et al., 2018). In addition, many microbe niches are present in microaerobic or anaerobic

conditions, such as underground, in sediments of environmental water, and in the digestive system of animals. It is, thus, essential to compare the conjugation features of plasmids under aerobic and anaerobic conditions to determine how plasmids spread among microbes in natural environments. Conjugation systems of plasmids in aerobes have been well-studied with several plasmids, including F, R388, and RP4/RK2 (Arutyunov and Frost, 2013, Grohmann et al., 2018, Getino and de la Cruz, 2018), and that of pCW3 has also been well-studied, hosted by the obligate anaerobe *Clostridium* (Wisniewski and Rood, 2017). Nevertheless, few studies have compared conjugation efficiency of the plasmid in the absence or presence of oxygen. Król et al. showed how FbFP protein, a fluorescence protein independent of oxygen, can detect conjugation under microaerobic or anaerobic conditions (Król et al., 2010). Notably, they showed that transfer frequencies of IncP-1 plasmid pB10 in both filter and liquid mating experiments were significantly lower under anaerobic conditions than under aerobic conditions using the above protein in *Escherichia coli* as donor and recipient (Krol et al., 2011). However, its fluorescence intensity is much lower than that of other marker proteins, including green fluorescence protein (GFP). It is also not suitable for use in various bacteria (Mukherjee et al., 2013).

The host ranges of plasmids are essential features for understanding how plasmids promote bacterial evolution and adaptation in various environments, including in relation to the occurrence of drug-resistant pathogens. Thus, plasmid host ranges are estimated qualitatively as narrow or broad via conjugation assays (Krishnan and Iyer, 1988, Shintani et al., 2005, Mierzejewska et al., 2007, Brown et al., 2013, Yanagiya et al., 2018). Some studies have conducted comprehensive analyses of the host ranges of

plasmids using the microbial communities of natural environmental samples, especially for incompatibility (Inc) P-1, P-7, P-9, and PromA plasmids (De Gelder et al., 2005, Shintani et al., 2014, Klumper et al., 2015, Li et al., 2018). Although the host range is intrinsic to the plasmid, the obtained range of transconjugants can change by different conditions, because under a certain environmental condition, some bacterial strains can be better recipients, whereas others can be worse. Comparing transconjugant ranges of plasmids under aerobic and anaerobic conditions is important to understand how the plasmids spread in natural environments, but to the best of our knowledge, no studies have compared transconjugant ranges between such conditions.

Here, the frequency of transconjugants for model plasmids was compared under aerobic and anaerobic conditions using GFP as a conjugation marker. Although GFP requires oxygen for development of the fluorophore (Reid and Flynn, 1997), its expression is not restricted under anaerobic conditions and its fluorescence is quickly recovered upon exposure to oxygen, a phenomenon known as aerobic fluorescence recovery (AFR) (Zhang et al., 2005, Pinilla-Redondo et al., 2018). Both incompatibility (Inc) P-1 (pBP136) and P-7 (pCAR1) plasmids were used as model plasmids, and two *Pseudomonas* donors (an obligate aerobe and facultative anaerobe) were used. Two facultative anaerobic *Enterobacteriaceae* strains and one *Pseudomonadaceae* strain were used as recipients because they could be easily selected with antibiotic resistance markers. In addition, pCAR1 is known as a narrow-host-range plasmid, which can be transferred and replicate only among genus *Pseudomonas* strains (Shintani et al., 2006, Shintani et al., 2014). The transconjugants ranges of these plasmids were also compared with candidates of recipients extracted from natural environmental samples.

Materials and Methods

Bacterial strains, plasmids, media, and culture conditions

The bacterial strains used in this study are listed in Table 1. Model plasmids, pBP136::*gfp* and pCAR1::*gfp* (Shintani et al., 2014, Kamachi et al., 2006, Maeda et al., 2003, Takahashi et al., 2009), were used. Bacterial strains were grown in Luria Broth (LB) (Sambrook and Russell, 2001). Antibiotics were used at final concentrations of 50 μg/mL for kanamycin (Km), 30 μg/mL for gentamicin (Gm), and 50 μg /mL for rifampicin (Rif). Solid medium was prepared by the addition of 1.5% (w/v) agar. For anaerobic conditions, 0.3 g/L L-cysteine (pH 7.0) and 1 mg/L resazurin (an indicator of oxygen) [the concentration of these compounds were based on GAM Agar, modified "Nissui" (Nissui Pharmaceutical Co., LTD., Tokyo, Japan) and Król et al., 2011] were added to the LB, which was designated as LB-ana. The solid media were prepared by the addition of 1.5% (w/v) agar to LB or LB-ana. For the cultivation of *Pseudomonas stutzeri* JCM 5965^T under anaerobic conditions, 5 g/L of KNO_3 was added to the LB-ana as an electron acceptor.

PBS or dH_2O with similar treatments with 0.3 g/L L-cysteine (pH 7.0) and 1 mg/L resazurin and N_2 bubbling were designated as PBS-ana or dH_2O-ana. After being bubbled with nitrogen (N_2) gas to remove dissolved oxygen, the resultant mixture was autoclaved. Medium plates were prepared in an anaerobic inoculation chamber (COY Laboratory Products, Inc. Grass Lake, MI, USA) consisting of a N_2/H_2 atmosphere. The plates were incubated at 30°C for two days before use. The agar plate without nutrients was prepared by mixing dH_2O or dH_2O-ana with 1.5% (w/v) agar and was named the 'Agar plate' or 'Agar-ana plate'.

Mating assays under aerobic and anaerobic conditions

(1) Mating assays with *Pseudomonas putida* SMDBS(pBP136::*gfp*) or *P. putida* SMDBS(pCAR1::*gfp*) as a donor

The donor and recipient strains were precultured under aerobic conditions at 30°C for 24 h (stationary phase) at 180 rpm for the donor and at 37°C and 200 rpm for the recipients in 8 mL LB liquid medium with appropriate antibiotics. After harvesting 4 mL (using two 2 mL tubes) under aerobic conditions (15,000×*g*, 2 min, 20°C), 2 mL of the donor and recipient were subjected to mating assays under aerobic conditions [the initial number of colony forming units (CFUs) of the donor or recipient are shown in Table S1]. The ratio of donor:recipient was approximately 1:1. Each pellet of donor and recipient cells was resuspended in 1 mL of PBS and mixed thoroughly by pipetting (washing the pellets). After harvesting again, the resultant pellet was resuspended in 130 µL LB. These cell mixtures were placed on 0.45 µm mixed cellulose ester filters (45 mm diameter, Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and the resultant filter was incubated on LB agar plates at 30°C for 3 and 24 h, respectively, in aerobic conditions [Aerobic-Aerobic (AE-AE), Figure 1].

The remaining half of the precultured cells was subjected to mating assays under anaerobic conditions. Each pellet was washed with PBS-ana and resuspended in 130 µL LB-ana. The cell mixtures were transferred to filter on LB-ana agar plates at 30°C for 3 or 24 h in an anaerobic chamber (N_2/H_2, the concentration of O_2 was below 100 ppm) [Aerobic/Anaerobic (AE-AN), Figure 1]. Regardless of aerobic or anaerobic filter mating, the mixtures on the filter were resuspended in 2 mL anaerobic PBS, and the appropriate dilutions of the cell suspension were spread on the LB-ana agar plates with Km (and

KNO_3 for transconjugants of *P. stutzeri*) in an anaerobic chamber (Figure 1). After detecting the colonies on the selective plates by incubating them in a chamber (30°C) for 2-3 d (no donor could grow under anaerobic condition), they were removed from the chamber and exposed to oxygen. Then, fluorescence (of GFP) was assessed for the transconjugant colonies (AFR). The number of donors, recipients, and transconjugants was determined by counting colonies on a respective selective medium. The frequency of transconjugants was expressed as the number of transconjugants per sum of the number of donors and recipients.

(2) Mating assays with *P. stutzeri* JCM 5965R(pBP136::*gfp*) or *P. stutzeri* JCM 5965R(pCAR1::*gfp*) as a donor

For mating assays between facultative anaerobes and the preparation of donors and recipients, mating on the filter and spreading on the selective plates (LB-ana with Km for transconjugants of *B. agrestis* and *P. agglomerans*, LB-ana with Km, Gm and KNO_3 for transconjugants of *P. stutzeri*) were performed similarly to the aforementioned method (Figure 1). These strains could be cultivated under anaerobic conditions, but *P. stutzeri* requires KNO_3 under anaerobic conditions. The donor and recipient strains were precultured with 8 mL LB-ana by statically incubating them in the anaerobic chamber at 30°C for 48 h [stationary phase, Anaerobic-Anaerobic (AN-AN) or Anaerobic-Aerobic (AN-AE), Figure 1]. Harvesting, washing, resuspension, filter mating, and spreading on the plates were also performed in an anaerobic chamber. Note that the donor strains could not grow on LB-ana without KNO_3 or LB-ana with Gm. The fluorescence of GFP in transconjugants was detected after exposure of the selective plates to oxygen.

Standard DNA manipulation

The total DNA of each strain (donors, recipients, and transconjugants) was extracted from each bacterial strain using a NucleoSpin® Tissue Kit (Macherey-Nagel). Total DNA from granules of a lab-scale upflow anaerobic sludge blanket (UASB) reactor (see section 2.4) and cow manure was extracted using a DNeasy PowerSoil Kit (Qiagen, GmbH, Hilden, Germany). Repetitive extragenic palindromic-PCR (BOX-PCR) was performed with primer BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′; (Versalovic et al., 1994, Shintani et al., 2011) and TaKaRa ExTaq® Polymerase (Takara Bio Inc. Shiga, Japan) under the following conditions: 95°C for 7 min followed by 30 cycles at 94°C for 1 min, 51°C for 1 min, then 65°C for 8 min. Then, the reactions were incubated at 65°C for 16 min and held at 16°C. The resultant products were subjected to agarose gel electrophoresis to check the transconjugants were derived of recipient strains by band patterns of each product.

The presence of plasmids in the transconjugant was confirmed by PCR amplification of the DNA region in each plasmid with PrimeSTAR® GXL (TAKARA BIO) or KOD One PCR MasterMix (Toyobo Co., Ltd., Osaka, Japan) with primers for *trfA-oriV* [pBP136, forward: 5′-TCAAGTGTCAGCACGGTAGG-3′, reverse: 5′-ATCAACGGCCGGTACTACAC-3′ (Shintani et al., 2014)] and *repA* [pCAR1, forward: 5′-TTGGGATTTACGGGACTGCT-3′, reverse: 5′-TCGGATGCCTATCAACGATT-3′ (Shintani et al., 2014)]. The conditions were: 30 cycles of 98°C for 10 s, 60°C for 15 s and 68°C for 1 min. Then, the reactions were held at 15°C for PrimeSTAR® GXL before 30 cycles at 98°C for 10 s, 55°C for 5 s, and 68°C for 5 s, after which they were again

held at 15°C. The amplified products were subjected to agarose gel electrophoresis to confirm their sizes.

Statistical analyses

The data for assessing the effect of oxygen on the transconjugant frequency for plasmids were analyzed using Student's *t*-tests or Welch's *t*-tests ($p < 0.05$) for aerobic donor and facultative anaerobic recipients (for Figure 2). The effects of the duration of mating were also analyzed using Student's *t*-tests or Welch's *t*-tests ($p < 0.05$). For transconjugant frequency between the facultative donor and recipient, data were analyzed using analysis of variance (ANOVA) with a post-hoc Tukey HSD (Honestly significant difference) test ($p < 0.05$) (for Figure 3).

*Comparisons of transconjugants range of pBP136::*gfp *under aerobic and anaerobic conditions*

The preparation of a donor strain culture, *P. putida* SMDBS(pBP136::*gfp*) was performed similarly to the aforementioned method under aerobic condition. Microbes in environmental samples, including granules from an anaerobic wastewater treatment plant and cow manure were used as recipient bacteria. The granules of the anaerobic wastewater plant were sampled from a lab-scale UASB reactor for methane fermentation (total volume 1 L) (Yanagiya et al., 2018, Suzuki et al., 2015) on May 6, 2017. The cow manure was sampled from cows that were not fed antibiotics in the Sumiyoshi field of the University of Miyazaki, Japan, on October 11, 2016. Microbial fractions from these samples were collected as follows: 1 g of granules of UASB or cow manure was

vigorously mixed in 10 mL of PBS (AE-AE) or PBS-ana (AE-AN). The resulting supernatant (130 µL) was used for recipient bacteria. The number of microbial cells in these samples was counted via microscopy after staining the cells with 4',6-diamidino-2-phenylindole (DAPI) or SYBR Green. Then, 2 mL of aerobic overnight culture of the plasmid donor in LB-medium was harvested, washed by PBS or PBS-ana, then resuspended in fresh PBS or PBS-ana. Around 10^8 CFU/mL of the donor suspended in 130 µL PBS or PBS-ana was mixed with 130 µL of 10^8~10^9 cells/mL bacteria extracted from the above environmental samples. The sample mixture was dropped on the mixed cellulose ester filters on the LB or LB-ana agar plates for 3 d at 30°C under aerobic or anaerobic condition. The mixture on the filter was resuspended with PBS, incubated at room temperature for an hour, then subjected to flow cytometry. The cell sorter MoFlo XDP® IntelliSort II instrument (Beckman Coulter, Denver, MA, USA) was equipped with a CyClone robotic arm for plate sorting using a 488 nm argon laser and 70 µm nozzle orifice. Sorting of each transconjugant cell was performed under the conditions previously described (Shintani et al., 2014). The gate for sorting transconjugants was set based on forward scatter and the intensity of the green fluorescent protein (GFP) fluorescence (Figure S1). To distinguish the cells of transconjugants from those of donor, the false-positive signals of cells or particles with autofluorescence in the environmental microbes were excluded by comparison between the samples with donor cells [*P. putida* SMDBS(pBP136::*gfp*)] and those without donor (Shintani et al., 2014). Each of the 384 cells was sorted on LB plates and incubated at 30°C for 2 d under aerobic condition to allow the cells to form a colony.

Sequencing of 16S rRNA genes of transconjugants

The obtained transconjugants were identified by the sequencing of a partial region of 16S rRNA genes amplified with 27F (5′-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3) using TaKaRa ExTaq or KOD One. The conditions were: 30 cycles at 98°C for 10 s, 55°C for 30 s, and 72°C for 60 s (ExTaq), after which the reactions were held at 15°C, or underwent 30 cycles at 98°C for 10 s, 55°C for 5 s, and 68°C for 5 s, then were held at 15°C (KOD One). The nucleotide sequences of the resultant PCR products were sequenced by the Sanger method using the 805R primer (5′-GACTACCAGGGTATCTAATC-3′). Identification of the genera of transconjugants was performed by BLAST with the EzBioCloud 16S database (Yoon et al., 2017) (Table 2).

The 16S rRNA gene amplicon sequencing of microbes in the granules of UASB and cow manure were performed on the total DNA of their microbial fractions before mating assays without donors. The first PCR was performed with a primer set of 515f-MIX (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA-3′) and 806r_MIX (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNGGACTACHVGGGTWTCTAAT-3′) using ExTaq HS (TAKARA BIO). The conditions were; 94°C for 2 min and 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, then 72°C for 5 min. After purification of the PCR products, the second PCR was performed with a primer set of 2ndF (5′- AATGATACGGCGACCACCGAGATCTACAC-Index2-ACACTCTTTCCCTACACGACGC-3′) and 2ndR (5′-

CAAGCAGAAGACGGCATACGAGAT-Index1-GTGACTGGAGTTCAGACGTGTG-3′) using ExTaq HS (TAKARA BIO). The nucleotide sequences were determined by MiSeq (2 x 300 bp, Illumina San Diego, CA, USA). The read sequences matching the primer sequence were extracted using the barcode splitter of the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and reads were trimmed with a quality threshold of >20 using sickle (https://github.com/najoshi/sickle). The reads with lengths of less than 40 bases were excluded from the analysis. The merge of the reads was performed with FLASH software (Magoc and Salzberg, 2011) (minimum overlap of 10 bases). The chimeric sequences and noises were removed by QIIME 2 (quantitative insights into microbial ecology), and an OTU was defined as a nucleotide sequence group that showed 97% identity. The above 246-260 base of 16S rRNA gene sequences were identified by Geneious Prime 2019 software (Kearse et al., 2012) with EzBioCloud database (Yoon et al., 2017) as the reference database.

Accession numbers of nucleotide sequence data

The partial sequences of 164 transconjugants were deposited in the DDBJ, EMBL, and GenBank databases (accession numbers LC509113-LC509281). The amplicon sequence data of 16S rRNA genes in granules of UASB and cow manure were deposited in the DDBJ Sequence Read Archive (DRA) with accession numbers DRA009069, DRA009496, DRA011101, and DRA011102.

Results

Effect of oxygen on transconjugant frequency

Mating assays were performed between the obligate aerobic donor *P. putida* SMDBS and facultative anaerobic recipients (*Buttiauxella agrestis* JCM1090^T, *Pantoea agglomerans* JCM 1236^T, and *P. stutzeri* JCM 5965^T) (Table 1, Figure 1). The transconjugant frequencies for pBP136::*gfp* from *P. putida* to facultative anaerobic recipients *P. agglomerans* or *P. stutzeri* were significantly lower (10^1-10^3 fold lower, $p < 0.05$) in AE-AN condition than in AE-AE condition, whereas those to *B. agrestis* were not (Figure 2A, Table S1-1). Similarly, a decrease in the transconjugant frequency in mating under anaerobic condition was observed for another plasmid, pCAR1 (Figure 2B, Table S1-1). These phenomena were observed regardless of the duration of conjugation (3 or 24 h) (Figure 2B).

For the conjugations between the facultative anaerobic donor and recipient strains, their transconjugant frequencies for pBP136::*gfp* were significantly lower when filter mating was performed under anaerobic condition compared to those under aerobic condition, which was observed for both 3 and 24 h mating assays ($p < 0.05$, Figure 3A-C, Table S1-2). Similar tendency was also observed if the mating assays were performed on Agar or Agar-ana plate (without nutrients) (Figure S2). For pCAR1::*gfp*, significant differences in transconjugant frequencies between AE-AE and other conditions were not observed in the 3 h but were in the 24 h mating assays ($p < 0.05$, Figure 3D, Table S1-2). Strikingly, the differences in the transconjugant frequencies of both plasmids between

AE-AN and AN-AN conditions were not significant in any mating assays (Figure 3A-D, Table S1-2). These results indicate that transconjugant efficiency was influenced by oxygen, coinciding with the results of a previous study (Krol et al., 2011). Considering the procedures of these mating assays (Figure 1), in many cases, the presence or absence of oxygen during mating influenced transconjugant frequencies. In contrast, in the case of mating assays under anaerobic condition, the presence of oxygen during preculture did not influence the frequencies.

Mating duration largely influenced transconjugant frequency under aerobic condition

Comparison of the transconjugant frequencies for pBP136::*gfp* under different durations of conjugation (3 and 24 h) showed that they were not significantly different to those of *P. putida* to *B. agrestis* ($p > 0.05$), whereas those for *P. putida* to *P. agglomerans* and to *P. stutzeri* were significantly higher in 24 h mating ($p < 0.05$) (Figure 2A, Table S1-3). Transconjugant frequencies for pCAR1::*gfp* were significantly higher in the AE-AE condition ($p < 0.05$) with 24 h mating but not under AE-AN condition (Figure 2B, Table S1-3).

Notably, transconjugant frequencies between *P. stutzeri* as a donor of pBP136::*gfp* to the recipients were significantly higher in 24 h rather than 3 h mating under AE-AE and AN-AE conditions ($p < 0.05$) but not consistently significant under AE-AN or AN-AN conditions ($p > 0.05$) (Figure 3ABC, Table S1-4). The frequencies of pCAR1::*gfp* were significantly higher for 24 h mating under AE-AE, AE-AN, and AN-AE conditions ($p < 0.05$) but not consistently significant under AN-AN condition (Figure

3D, Table S1-4). These findings collectively highlight that a longer duration of filter mating does not increase transconjugant frequencies under anaerobic condition.

Different transconjugant were obtained under aerobic and anaerobic conditions

As shown in Figure 2, the degree of reduction in transconjugant frequencies via mating under anaerobic condition could change due to different combinations of the donor and recipients, such as the reduction observed from *P. putida* to *P. agglomerans* at around 10^{-3} in 24 h mating, yet that from *P. putida* to *P. stutzeri* was around 10^{-1} in 24 h mating (Figure 2A). This resulted in the rank of the transconjugant frequency being reversed by the presence or absence of oxygen. The frequency was higher from *P. putida* to *P. agglomerans* than from *P. putida* to *P. stutzeri* under AE-AE condition; however, it was lower in AE-AN condition (Figure 2A). Therefore, it is possible that the transconjugant ranges of a plasmid, which was defined as the maximum phylogenetic distance among transconjugants in the present study, could change under aerobic and anaerobic conditions. Following this investigation, the transconjugant ranges of pBP136::*gfp* from *P. putida* to other recipients were compared under aerobic and anaerobic conditions. Microbes extracted from different environmental samples, including anaerobic granules from a UASB and cow manure were used as recipients. After mating with the aerobically cultured donor cells under aerobic and/or anaerobic conditions (on LB or LB-ana plates), cells of transconjugants displaying green fluorescence were collected by flow cytometry and cell sorting under aerobic condition (Table 2). The frequency of fluorescent cells after mating under aerobic condition was 10^2-fold higher than those under anaerobic condition, indicating that the numbers of transconjugant cells were lower by anaerobic

mating than aerobic mating; for the bacteria from granules of UASB and cow manure, 0.19% and 0.73% of particles, including donor and recipient cells, were detected as putative transconjugants obtained under aerobic condition, while 0.0047% and 0.0057% of particles were detected under anaerobic condition (Figure S1). A total of 164 transconjugants were obtained, all of which had plasmid pBP136::*gfp* (Table 2). Thirteen genera (*Paracoccus*, *Rhizobium*, *Advenella*, *Caenimicrobium*, *Candidimonas*, *Orrella*, *Pusillimonas*, *Hydrogenophaga*, *Acinetobacter*, *Stenotrophomonas*, *Haemophilus*, *Bacillus*, and *Flavobacterium*) in three different phyla were uniquely identified as transconjugants under aerobic condition. In contrast, three genera (*Buttiauxella*, *Klebsiella* and *Phytobacter*) in *Gammaproteobacteria* were unique under anaerobic conditions (Table 2). Notably, transconjugants belonging to *Gammaproteobacteria* (family *Enterobacteriaceae* and genus *Pseudomonas*) was obtained by mating under anaerobic condition, while no other bacteria including *Alphaproteobacteria*, *Betaproteobacteria* were found (Table 2). These results suggest that a plasmid could differently spread among bacteria under different concentrations of oxygen.

Shift of microbial communities under aerobic and anaerobic conditions partially affects transconjugant range

To assess whether the differences in transconjugant range between aerobic and anaerobic conditions were due to shifts of recipient communities during mating, microbial communities were compared based on 16S rRNA gene amplicon sequencing. The comparisons were performed between extracted bacteria (recipients) from environmental samples (granule of UASB and cow manure) before mating (BM in Figure 4) and those after mating (AM), incubated on LB plate in the absence or presence of donors [AM(-) or

AM(+) in Figure 4]. The composition of microbes in the AM samples contained three major phyla; *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* in both microbes from granule of UASB and cow manure regardless of the absence or presence of donors (Figure 4A). At the class levels of them, there were clear shifts of microbial communities from BM to AM samples both during aerobic and anaerobic conditions (Figure 4BCD). Among *Proteobacteria*, *Gammaproteobacteria* was most abundant class in AM samples both under aerobic and anaerobic conditions in microbes of granule of UASB and cow manure (Figure 4B). *Betaproteobacteria* was abundant in AM samples under aerobic than anaerobic condition in both microbes (Figure 4B). *Alphaproteobacteria* was less abundant in AM samples in microbes of granule of UASB, while that was found in AM samples in microbes of cow manure under aerobic condition (Figure 4B). Shifts of microbial communities were also clearly found in family levels in *Gammaproteobacteria* from BM to AM samples (Figure 4E). Classes including *Enterobacteriaceae*, *Moraxellaceae*, *Pseudomonadaceae*, or *Xanthomonadaceae* were more abundant in AM than in BM samples (Figure 4E). As for the classes or orders in *Betaproteobacteria* and *Alphaproteobacteria*, there were also clear shifts of microbial communities from BM to AM samples (Figure 4FG). These results suggested that the shift of recipient communities occurred during mating on LB under aerobic or anaerobic condition.

Discussion

The present study identified three important phenomena that occur when conjugation takes place under anaerobic condition: (i) oxygen is not required for the conjugation of plasmids, (ii) the presence of oxygen during conjugation affects the efficiency of conjugation, and (iii) different transconjugants of plasmid are obtained under aerobic and

anaerobic conditions. The former two phenomena were shown with IncP-1 (pBP136) and IncP-7 (pCAR1) plasmids, while the last phenomenon was shown with only the IncP-1 plasmid. These results indicate that the presence of oxygen during filter mating could influence the syntheses of conjugative machinery. The machinery includes MOB proteins, type IV coupling proteins, and type IV secretion system pili, that are involved in DNA mobility, the physical contact between the donor and recipient cells, and the secretion of plasmid DNA. Comparisons of transcriptional levels of the genes encoding the machinery would be necessary for the further study. Both procedures require ATP in donor cells (Gomis-Ruth et al., 2001, Christie et al., 2014). Anaerobes can produce ATP both under aerobic and anaerobic conditions by different ways, but under aerobic conditions (AE-AE and AN-AE conditions), donor cells could efficiently produce ATP during filter mating, which may explain the higher conjugation frequency than that of the AE-AN and AN-AN conditions (Figures 2 and 3). Moreover, it could also explain why a longer duration of filter mating under aerobic condition increased the conjugation frequency (AE-AE in Figure 2, and AE-AE and AN-AE conditions in Figure 3).

On the other hand, *P. stutzeri* synthesizes ATP via denitrification in the presence of nitrogen oxides (Lalucat et al., 2006). In the present study, filter mating under anaerobic condition was performed on LB-ana without KNO_3. Thus, theoretically, neither donor *P. putida* nor *P. stutzeri* can produce ATP during filter mating under anaerobic condition. Henceforth, neither donor grew on LB-ana plates without KNO_3 under anaerobic condition. Indeed, no colonies of these donors were detected on LB-ana plates without KNO_3 (data not shown). This may be why the transconjugant frequency did not

change between 3 and 24 h mating under either AE-AN or AN-AN condition (Figures 2 and 3).

There have been some reports showing that oxygen is required for efficient transfer of IncP-1 plasmids (Shoemaker et al., 1986a, Krol et al., 2011). Interestingly, Shoemaker et al. showed that the frequency of IncP-1 plasmid transfer from *E. coli* donors to *Bacteroides* recipients was at least 50 times higher under aerobic condition than anaerobic condition (Shoemaker et al., 1986a). However, when *Bacteroides* was used as donor, the transfer frequency to *E. coli* was the same under both conditions, although the absolute number of transconjugants was 3-5 times higher in aerobic condition (Shoemaker et al., 1986b). Król et al. reported that oxygen availability was probably important for high density of donor and recipient cells (Krol et al., 2011). These facts suggested that the density of donor and recipient cells would be a key factor for efficient transfer of plasmid. Indeed, during the filter mating process, donors, recipients, and transconjugants grew and propagated under aerobic condition. For anaerobic conjugation, recipients and transconjugants except *P. stutzeri* grew and propagated on LB-ana plates without KNO_3 as they could form colonies (data not shown). Therefore, the transconjugant frequency could change by extending the duration of filter mating. Notably, the number of donor and recipient strains during mating did not always drastically change compared to those before mating, whereas those of transconjugants did (Table S1-1). This suggests two possibilities: (i) the number of conjugation events between donor and recipient cells or between transconjugants to another recipient cell increases by extending the duration of filter mating, (ii) transconjugants grow and propagate during filter mating, but growths of donor and recipient cells could not be

accurately measured, because large numbers of donor and recipient strains (10^8-10^{10} CFU/mL) requires growth nutrients further more than transconjugant strains (10^4-10^5 CFU/mL) (Table S1-1). For the second possibility, instead of an LB plate, filter mating using *P. stutzeri* as the donor and recipient strain was performed on Agar or Agar-ana plates without nutrients, on which no strains grew or propagated. The transconjugant frequencies under AE-AN and AN-AN conditions did not significantly increase by extending the duration of mating ($p > 0.05$, 3 to 24 h), respectively, whereas those under AE-AE increased ($p < 0.01$) (Table S1-1 and Figure S2). This result indicates that the transconjugants did not grow or propagate during filter mating and, thus, the second possibility might be excluded. Therefore, it is possible that the number of conjugation events from donor to recipient cells may increase by extending the duration of filter mating, especially under aerobic condition (the first possibility).

Different transconjugants of pBP136 were obtained by mating with microbes extracted from granule of UASB and cow manure, or under aerobic and anaerobic conditions (Table 2). Whereas wide range of transconjugants were obtained by mating under aerobic condition, only limited range and small number of transconjugants were obtained under anaerobic condition (Table 2). Additional filter mating assays were performed with microbes extracted from another cow manure samples (cow manure #2, Supplemental text). A longer period of time was spent sorting cells with fluorescence in the samples under anaerobic conditions. The mating assays were also performed on Agar or Agar-ana plates. A total of 199 colonies under aerobic (100) and anaerobic (99) conditions were successfully obtained as transconjugants, all of which had plasmid pBP136::*gfp* (Table S2). Although similar genera of *Proteobacteria* were obtained, but

no genera were uniquely identified as transconjugants under anaerobic condition (Table S2). Notably, two genera, *Escherichia* and *Shigella*, were majorly obtained both under aerobic and anaerobic conditions (Table S2), which were not obtained from microbes extracted from the previous cow manure sample (Table 2). This was probably because of differences in microbial communities between them (Figure S3). In any cases, one clear difference was that no transconjugants belonging to *Betaproteobacteria* or *Alphaproteobacteria* were obtained under anaerobic condition in both microbes (Table 2, S2). One of possible reasons of this difference was: the host of plasmid specifically detected under aerobic condition could also obtain the plasmid by mating under anerobic condition, but their frequency was below the detection limit and thus we could not detect them. Another possible reason was that the abundance of potential hosts changed during mating assays under aerobic or anaerobic condition for three days as shown in Figure 4. Some potential hosts in these classes could not survive under anaerobic condition for a long period. Indeed, the ratio of these classes were less abundant under anaerobic condition in both microbes (Figure 4B). Similarly, ratio of obligate aerobes and obligate anaerobes could change under aerobic or anaerobic condition. Indeed, *Clostridium* (family *Clostridia* of *Firmicutes*) are known to be very sensitive to low concentration of oxygen (Edwards et al., 2013, Edwards et al., 2016), and their abundance was higher in anaerobic condition than aerobic condition (Figure 4C). It should be noted that the obtained transconjugant ranges had limitations because their detection depends on the modified-*lac* promoter driven *gfp*. The transconjugants with *lacI* genes on their chromosome, and/or in which the *lac* promoter was not functioned under aerobic or

anaerobic condition were not likely to be detected. These points should be further analyzed.

Four *Buttiauxella* strains were uniquely obtained as transconjugants in microbes extracted from cow manure under anaerobic condition (Table 2). Considering that the transconjugant frequency by mating with donor *P. putida* and recipient *Buttiauxella* was not significantly different under AE-AE and AE-AN conditions (Figure 2A), the relative frequency of *Buttiauxella* as obtained transconjugants from microbes of cow manure could be higher after mating under anaerobic condition. On the other hand, *Pseudomonas* were obtained as transconjugants from microbes of granule of UASB and cow manure by mating both under aerobic and anaerobic conditions, while the frequency was lower under anaerobic condition (Table 2). Considering the fact that transconjugant frequency by mating with donor *P. putida* and recipient *Pseudomonas* were much lower (around 10^{-2}) under AE-AN condition than AE-AE condition (Figure 2A), the relative frequency of *Pseudomonas* could be high and be detected as transconjugants obtained from microbes of granule of UASB or cow manure (Table 2).

It should be noted that further in-depth analyses are required to understand how the difference in range of transconjugants occurred under aerobic and anaerobic conditions. Stecher et al. reported that conjugative transfer of a plasmid from *Salmonella* could be boosted by changes in communities of the gut microbes because the transfer was blocked by the commensal microbes other than *Enterobacteriaceae* strains (Stecher et al., 2012). Thus, comparisons of microbial communities will be important, not only before the conjugation assays, but also after growth under aerobic and anaerobic conditions.

In summary, the frequency and range of transconjugant could differ due to different concentrations in oxygen. The mechanisms of how they differ remain unclear, and thus, further detailed analyses at the molecular level will lead to a better understanding of the mechanisms of how the conjugation efficiency differs under different oxygen concentrations. Our findings indicate that a plasmid can differently spread among bacteria depending on the oxygen concentrations of the environment, which should be taken into account when predicting how plasmids and their accessory genes are disseminated in natural environments.

Author Contributions

MS conceived, designed, and supervised the study. KO, MT, KY, MY, and MS performed the experiments and data analysis. KO, MT, CSK, HN, MY, MO, KK, and MS wrote, reviewed, and edited the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Disclosure statement

The authors declare no conflict of interest.

Data Availability Statements

The data underlying this article are available in the article and in its online supplementary material.

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
Bacterial strains		
Escherichia coli S17-1 λ*pir*	RK2 *tra* regulon; host for *pir*-dependent plasmids; *recA thi pro hsdR* M RP4-2-Tc::Mu-Km::Tn*7* λ*pir* Tp^r Sm^r	(Simon et al., 1983)
Buttiauxella agrestis JCM 1090^T	Type strain	RIKEN, BRC-JCM
Buttiauxella agrestis JCM 1090R	Spontaneous Rif^r strain of JCM 1090^T	This study
Pantoea agglomerans JCM 1236^T	Type strain	RIKEN, BRC-JCM
Pantoea agglomerans JCM 1236R	Spontaneous Rif^r strain of JCM 1236^T	This study
Pseudomonas putida SMDBS	A *dapB* (encoding dihydrodipicolinate reductase, an essential enzyme for lysine synthetic)-deleted strain of *Pseudomonas putida* SM1443, which was Rif^r of KT2440 with mini-Tn*5*-*lacI*^{q} cassette inserted into the chromosome	(Shintani et al., 2014)

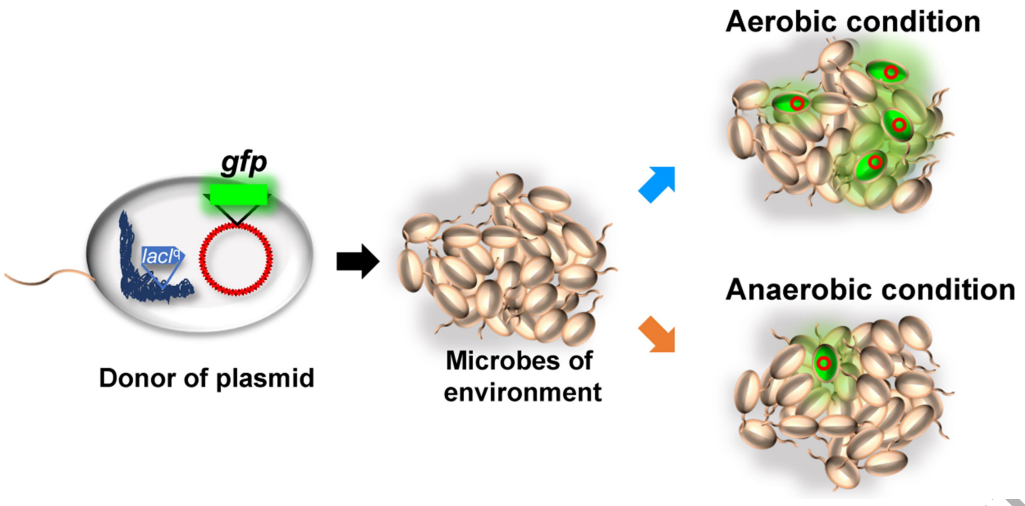
Pseudomonas putida SMDBS(pBP136::*gfp*)	SMDBS bearing pBP136::*gfp*	(Shintani et al., 2014)
Pseudomonas putida SMDBS(pCAR1::*gfp*)	SMDBS bearing pCAR1::*gfp*	(Shintani et al., 2014)
Pseudomonas stutzeri JCM 5965^{T}	Type strain	RIKEN, BRC-JCM
Pseudomonas stutzeri JCM 5965R	Spontaneous Rif^{r} strain of JCM 5965^{T}	This study
Pseudomonas stutzeri JCM 5965R(pBP136::*gfp*)	JCM 5965R bearing pBP136::*gfp*	This study
Pseudomonas stutzeri JCM 5965R(pCAR1::*gfp*)	JCM 5965R bearing pCAR1::*gfp*	This study
Pseudomonas stutzeri JCM 5965RG	JCM 5965R with mini-Tn*5*-Gm^{r} gene on pBSL202 was inserted into the chromosome	This study
Plasmids		
pBP136::*gfp*	pBP136 carrying Km^{r} and $P_{A1/O4/O3}$-*gfp* cassette in *parA*	(Shintani et al.,

	(26,137 nt)	2014)
pCAR1::*gfp*	pCAR1 carrying Km^r and $P_{A1/O4/O3}$-*gfp* cassette in ORF171 (182,625 nt)	(Shintani et al., 2014)
pBSL202	Ap^r, Gm^r mini-Tn*5*	(Alexeyev et al., 1995)

Table 2. Genera of transconjugants obtained by mating with microbes from environmental samples under aerobic (AE) and anaerobic (AN) conditions.

Top hit[a] (Genus level)	Total		granule of UASB		cow manure		Family	Order	Class	Phylum
	AE	AN	AE	AN	AE	AN				
Paracoccus	**12**	**0**	0	0	12	0	*Rhodobacteraceae*	*Rhodobacterales*	*Alpha-proteobacteria*	*Proteobacteria*
Rhizobium	**39**	**0**	39	0	0	0	*Rhizobiaceae*	*Rhizobiales*		
Advenella	**7**	**0**	0	0	7	0	*Alcaligenaceae*	*Burkholderiales*	*Beta-proteobacteria*	
Caenimicrobium	**8**	**0**	0	0	8	0				
Candidimonas	**9**	**0**	0	0	9	0				
Orrella*	**9**	**0**	0	0	9	0				
Pusillimonas	**1**	**0**	0	0	1	0				
Hydrogenophaga*	**1**	**0**	0	0	1	0	*Comamonadaceae*			
Buttiauxella	**0**	**4**	0	0	0	4	*Enterobacteriaceae*	*Enterobacteriales*	*Gamma-proteobacteria*	
Klebsiella	**0**	**3**	0	3	0	0				
Phytobacter	**0**	**5**	0	5	0	0				
Acinetobacter	**1**	**0**	0	0	1	0	*Moraxellaceae*	*Pseudomonadales*		
*Pseudomonas**	**30**	**3**	13	1	17	2	*Pseudomonadaceae*			
Stenotrophomonas	**26**	**0**	26	0	0	0	*Xanthomonadaceae*	*Xanthomonadales*		
Haemophilus	**2**	**0**	0	0	2	0	*Pasteurellaceae*	*Pasteurellales*		
Bacillus	**1**	**0**	0	0	1	0	*Bacillaceae*	*Bacillales*	*Bacilli*	*Firmicutes*
Flavobacterium*	**3**	**0**	0	0	3	0	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteriia*	*Bacteroidetes*
Total	**149**	**15**	**78**	**9**	**71**	**6**				

[a]Genera of transconjugants that showed >97% identity in the BLAST search with EzTaxon server 2.1 (23, www.eztaxon.org/). Genera uniquely obtained under aerobic (AE) condition are shown in red, while those obtained under anaerobic (AN) condition are shown in blue. '*' indicates that at least one transconjugant in the genus showed <97% identity.



Caption of Graphical Abstract

Frequency and range of transconjugants of plasmids in microbial communities could be different under aerobic or anaerobic conditions.

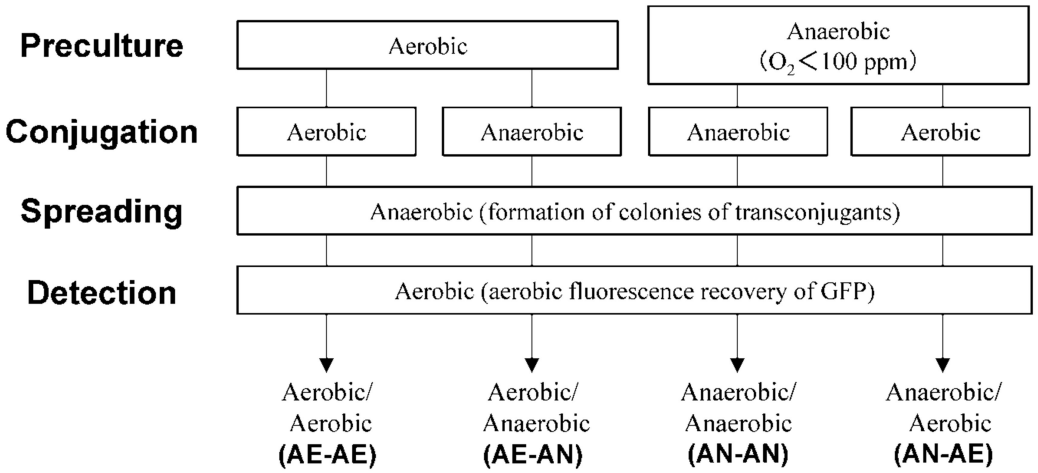


Figure 1. Schematic showing the experimental procedure. Using obligate aerobic donors, filter mating assays were performed with aerobically cultured (preculture) donors and recipients under aerobic and/or anaerobic conditions (AE-AE and AE-AN). Using facultative anaerobic donors and recipients, four combinations of filter mating assays were performed with aerobically or anaerobically cultured donors and recipients under aerobic and anaerobic conditions (AE-AE, AE-AN, AN-AN, and AN-AE).

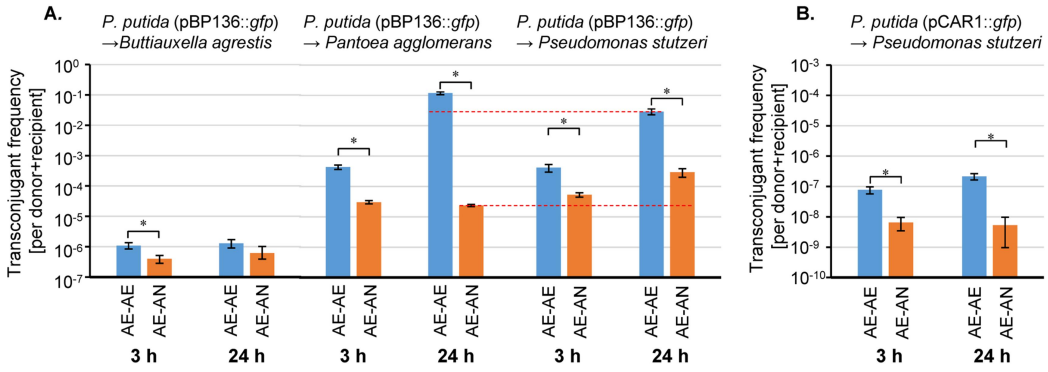


Figure 2. Transconjugant frequency for pBP136::*gfp* (panel A) and pCAR1::*gfp* (panel B) from the obligate aerobic donor *P. putida* SMDBS to the facultative anaerobic recipients (*Buttiauxella agrestis* JCM 1090R, *Pantoea agglomerans* JCM 1236R, and *P. stutzeri* JCM 5965RG) of 3 and 24 h mating assays. Each experimental procedure is shown in Figure 1. The error bars indicate standard deviations of triplicate experiments. Asterisks indicate statistical significance ($p < 0.05$, *t*-test, n = 3).

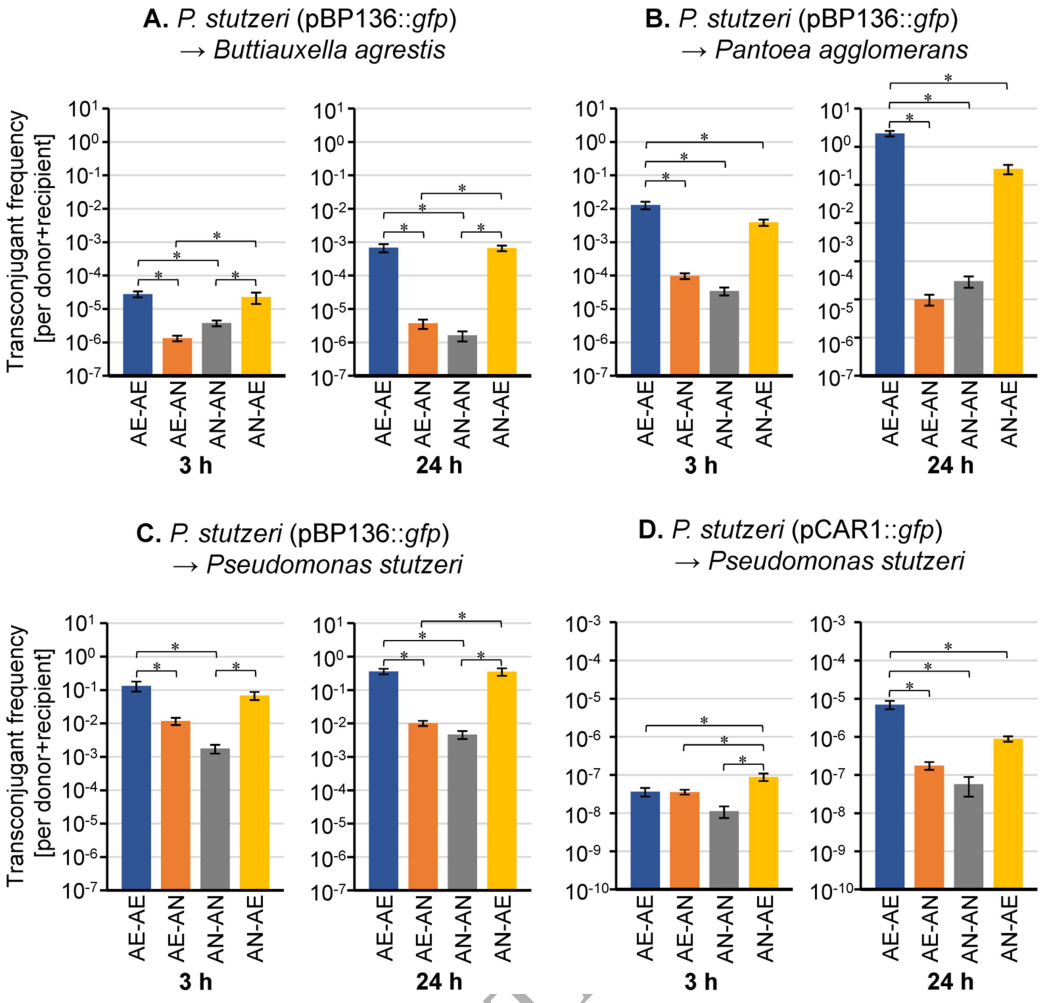


Figure 3. Transconjugant frequency for pBP136::*gfp* (panels A-C) and pCAR1::*gfp* (panel D) from the facultative anaerobic donor *P. stutzeri* JCM 5965R to the facultative anaerobic recipients (*Buttiauxella agrestis* JCM 1090R, *Pantoea agglomerans* JCM 1236R, and *P. stutzeri* JCM 5965RG) of 3 and 24 h mating assays. Each experimental procedure is shown in Figure 1. The error bars indicate standard deviations of triplicate experiments. Asterisks indicate significant differences (Tukey's HSD test, $p < 0.05$, n = 3).

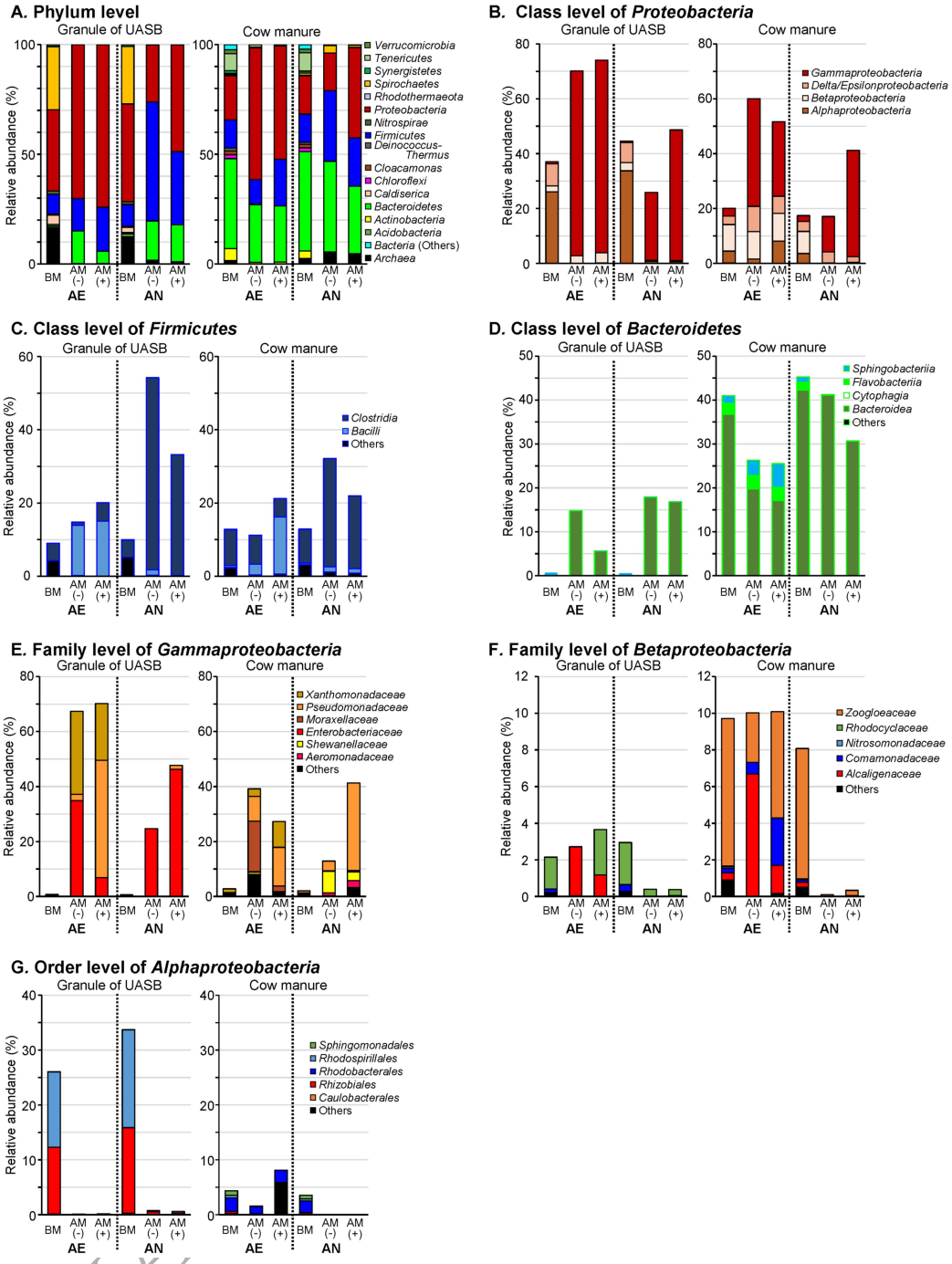


Figure 4. Microbial communities extracted from granules of UASB and cow manure before the mating assay (BM), and after the mating (AM) without donor cells (-) and with donor cells (+). 'AE' and 'AN' indicate aerobic and anaerobic conditions for mating assays. Panel A shows the microbial

communities at the phylum level (except for *Archaea*). Panels B-D show the microbial communities at the class level of *Proteobacteria*, *Firmicutes* and *Bacteroidetes*. Panels E-G show the microbial communities in family or order levels of *Proteobacteria*.

Supplementary Material

Oxygen concentration affects frequency and range of transconjugants for the incompatibility (Inc) P-1 and P-7 plasmids pBP136 and pCAR1

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The Supplementary information includes

- ✓ Supplemental text
- ✓ Figures S1-S3
- ✓ Table S1-S2

Supplemental text

Another cow manure was sampled from the same filed of the University of Miyazaki, Japan, on November 7, 2018 (named as cow manure #2). Microbial fractions from the samples were collected as described in the main text. The filter mating with donors were performed on the mixed cellulose ester filters on the LB or LB-ana agar plates or on the Agar plate or Agar-ana plate for 3-5 d at 30°C under aerobic or anaerobic conditions. The mixture on the filter was resuspended with PBS, incubated at room temperature for an hour, then subjected to flow cytometry. The obtained transconjugants were identified based on their partial 16S rRNA gene sequences listed Table S2. Their 16S rRNA gene sequences were deposited in in the DDBJ, EMBL, and GenBank databases (accession numbers LC509282-LC509433 and LC548661-LC548707). The presence of pBP136::*gfp* in each transconjugant was confirmed by PCR as described in main text. The 16S rRNA gene amplicon sequencing of microbes in the cow manure #2 were performed on the total DNA of their microbial fractions before mating assays without donors. The amplicon sequence data of 16S rRNA genes in cow manure were deposited in the DDBJ Sequence Read Archive (DRA) with accession numbers DRA009496. The comparisons of microbial communities of two cow manure samples were shown in Figure S3.

identity. The amplicon sequence data of 16S rRNA genes in granules of UASB and cow manure were deposited in the DDBJ Sequence Read Archive (DRA) with accession numbers DRA009069 and DRA009496.

References

Magoc, T., and Salzberg, S.L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957-2963.

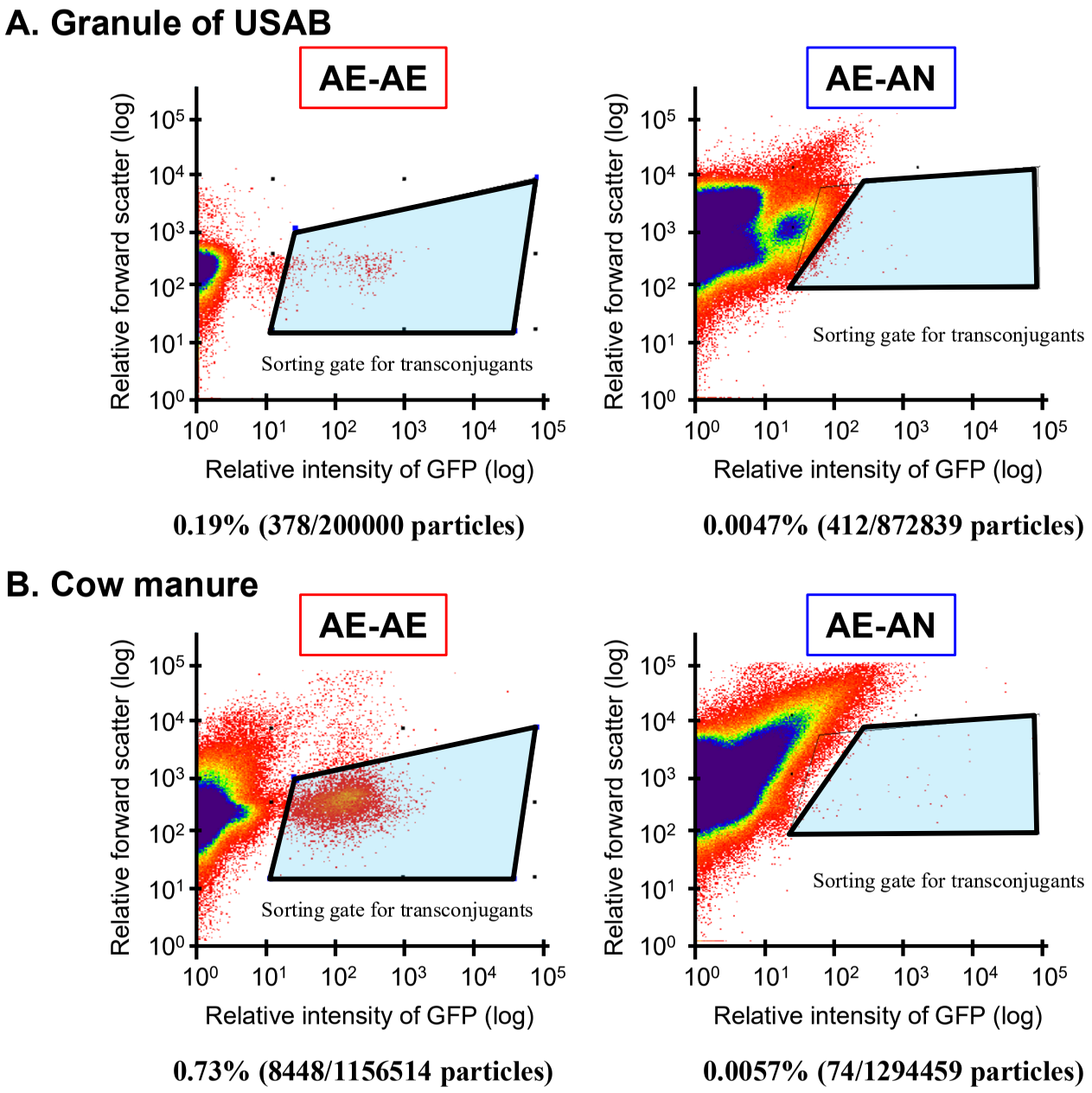

Figure S1. Scatter plots of particles in the samples after conjugation under AE-AE and AE-AN conditions.

The plots of conjugation with microbes in granule of UASB on LB (A), and those in cow manure on LB plate (B) are shown. The x- and y-axes were shown in logarithm whose base was 10. The sorting gates for transconjugant cells and the ratio of the numbers of particles in the gate to those of total particle are shown.

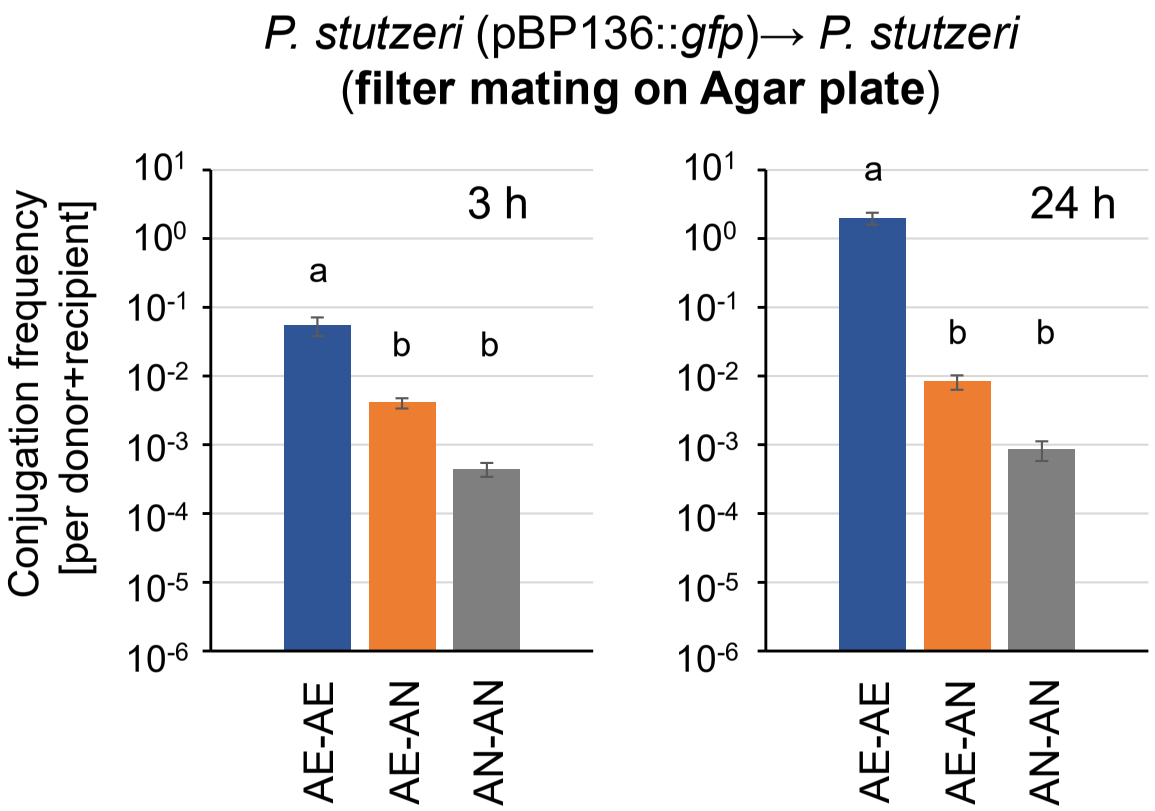

Figure S2. Conjugation frequency of pBP136::*gfp* from *P. stutzeri* JCM 5965R to *P. stutzeri* JCM5965R on Agar or Agar-ana plate.

The error bars indicate standard deviations of triplicate experiments. 'a', and 'b' indicate the significant differences (Tukey HSD test, $p < 0.05$, n = 3) in each duration of mating. The conjugation frequencies under AE-AN and AN-AN did not statistically significantly increase by extending the duration of mating (Student's *t* test, $p>0.05$, 3 h to 24 h), whereas those under AE-AE increased (Student's *t* test, $p<0.01$) .

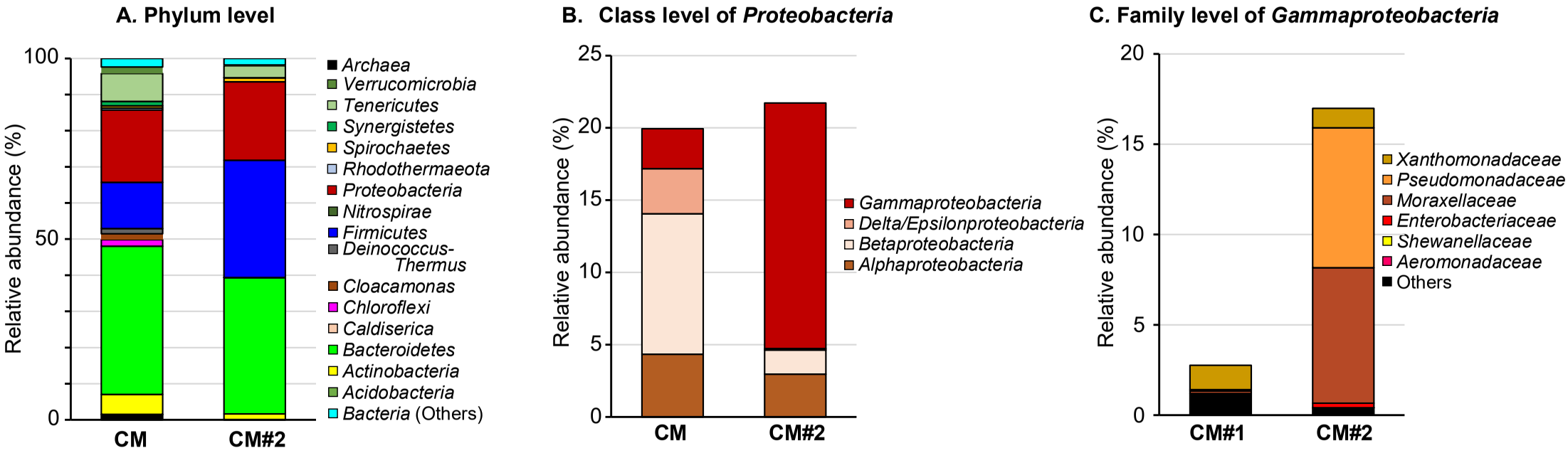

Figure S3. Microbial communities extracted from two cow manure samples (CM and CM#2) before the mating.

Panel A shows the microbial communities at the phylum level. Panels B and C show the microbial communities at the Class level of *Proteobacteria* and family level of *Gammaproteobacteria*, respectively.

Table S1-1. The numbers of donor, recipient, and transconjugants in the mating assays.

Donor	Recipient	Mating duration	Before filter mating						After fitler mating				Transconjugant frequency [T/D+R]	Standard deviations	*p*-value in *t* test
			Condition	Donor	Standard deviations	Recipient	Standard deviations	Donor [D]	Standard deviations	Recipient [R]	Standard deviations	Transconjugants [T]	Standard deviations			
Pseudomonas putida SMDBS(pBP136::*gfp*)	*Buttiauxella agrestis*	3 h	AE-AE	1.58.E+10	5.89.E+06	1.47.E+10	9.59.E+06	2.23E+09	3.67E+08	6.80E+09	5.57E+08	9.87E+03	1.29E+03	1.09E-06	2.55E-07	1.23E-02
			AE-AN	1.58.E+10	5.89.E+06	1.47.E+10	9.59.E+06	9.27E+08	5.69E+07	8.07E+09	8.50E+08	3.57E+03	6.51E+02	3.97E-07	1.12E-07	
		24 h	AE-AE	3.15.E+09	9.15E+08	1.33.E+10	3.29.E+09	8.67.E+09	1.46E+09	7.67.E+09	7.51E+08	2.12E+04	3.64E+03	1.30E-06	3.99E-07	6.21E-02
			AE-AN	3.15.E+09	9.15E+08	1.33.E+10	3.29.E+09	4.40E+09	6.56E+08	6.50E+09	1.35E+09	6.73E+03	1.23E+03	6.18E-07	2.27E-07	
	Pantoea agglomerans	3 h	AE-AE	1.63.E+09	1.14.E+08	4.85.E+09	8.23.E+08	8.37E+08	6.03E+07	2.94E+09	1.08.E+08	1.64.E+06	1.98.E+05	4.33E-04	7.16E-05	6.25E-04
			AE-AN	1.63.E+09	1.14.E+08	4.85.E+09	8.23.E+08	9.17E+08	9.29.E+07	2.96E+09	7.00.E+07	1.18.E+05	9.54.E+03	3.04E-05	3.74E-06	
		24 h	AE-AE	5.00.E+09	6.32.E+08	8.80.E+09	7.12.E+08	2.47E+09	1.98E+08	4.57E+08	1.10.E+08	3.45.E+08	6.51E+02	1.18E-01	1.24E-02	7.95E-05
			AE-AN	5.00.E+09	6.32.E+08	8.80.E+09	7.12.E+08	5.93E+08	3.21.E+07	3.56E+09	2.73.E+08	9.80E+04	6.51E+02	2.36E-05	1.89E-06	
	Pseudomonas stutzeri	3 h	AE-AE	3.70.E+09	1.29.E+09	1.67.E+10	1.36.E+09	8.53.E+08	7.09E+07	1.25.E+10	1.42.E+09	5.53.E+06	9.29.E+05	4.14E-04	1.16E-04	5.73E-03
			AE-AN	3.70.E+09	1.29.E+09	1.67.E+10	1.36.E+09	7.40.E+08	6.56.E+07	1.46.E+10	8.08.E+08	8.17.E+05	8.50.E+04	5.31E-05	8.55E-06	
		24 h	AE-AE	2.60.E+09	3.65.E+08	8.30.E+09	1.11.E+09	4.08.E+09	1.06.E+08	3.43.E+09	4.31.E+08	2.21.E+08	3.26.E+07	2.94E-02	6.44E-03	1.44E-03
			AE-AN	2.60.E+09	3.65.E+08	8.30.E+09	1.11.E+09	3.77.E+08	3.36.E+07	8.27.E+09	1.04.E+09	2.53.E+06	4.73.E+05	2.93E-04	9.11E-05	
Pseudomonas stutzeri JCM 5695(pBP136::*gfp*)	*Buttiauxella agrestis*	3 h	AE-AE	1.77.E+10	1.63.E+09	6.25.E+09	9.57.E+08	1.51.E+10	1.25.E+09	9.47.E+09	8.50.E+08	6.87.E+05	8.08.E+04	2.80E-05	5.68E-06	NA
			AE-AN	1.77.E+10	1.63.E+09	6.25.E+09	9.57.E+08	1.48.E+10	1.31.E+09	7.80.E+09	6.56.E+08	3.01.E+04	3.20.E+03	1.33E-06	2.57E-07	
			AN-AN	2.19.E+09	1.81.E+08	5.70.E+09	7.75.E+08	2.78.E+09	1.12.E+08	4.90.E+08	8.00.E+07	1.23.E+04	1.71.E+03	3.77E-06	7.44E-07	
			AN-AE	9.50.E+09	1.59.E+09	4.20.E+09	1.03.E+09	1.16E+10	6.66.E+08	7.37.E+09	1.67.E+09	4.30.E+05	1.06.E+05	2.26E-05	8.35E-06	
		24 h	AE-AE	1.47.E+10	1.39.E+09	1.20.E+10	1.97.E+09	1.11.E+10	1.89.E+09	9.53.E+09	1.03.E+09	1.42.E+07	1.87.E+06	6.85E-04	1.87E-04	
			AE-AN	1.47.E+10	1.39.E+09	1.20.E+10	1.97.E+09	9.37.E+09	1.89.E+09	9.80.E+09	9.17.E+08	7.07.E+04	1.20.E+04	3.69E-06	1.17E-06	
			AN-AN	1.19.E+10	2.17.E+09	1.26.E+10	1.57.E+09	7.23.E+09	1.53.E+09	5.97.E+09	1.07.E+09	2.130E+04	2.95.E+03	1.61E-06	5.42E-07	
			AN-AE	9.50.E+09	1.59.E+09	4.20.E+09	1.03.E+09	1.32E+10	9.45.E+08	9.43.E+09	6.51.E+08	1.510E+07	1.80.E+06	6.66E-04	1.26E-04	
	Pantoea agglomerans	3 h	AE-AE	1.77.E+10	1.47.E+09	1.73.E+10	8.08.E+08	1.16.E+09	1.66.E+08	1.01.E+09	1.88.E+08	2.82.E+07	2.57.E+06	1.30E-02	3.29E-03	NA
			AE-AN	1.77.E+10	1.47.E+09	1.73.E+10	8.08.E+08	1.31.E+10	1.21.E+09	1.49.E+09	1.45.E+08	1.43.E+06	1.46.E+05	9.82E-05	1.91E-05	
			AN-AN	3.70.E+09	4.76.E+08	1.29.E+08	9.59.E+06	9.70.E+08	9.54.E+07	6.17.E+08	1.53.E+07	5.50.E+04	1.08.E+04	3.47E-05	9.24E-06	
			AN-AE	1.44.E+10	2.32.E+09	1.45.E+10	1.75.E+09	3.17.E+09	4.73.E+08	4.60.E+09	7.00.E+08	3.04.E+07	1.82.E+06	3.92E-03	8.26E-04	
		24 h	AE-AE	1.53.E+10	4.43.E+08	1.26.E+10	5.74.E+08	1.95.E+09	2.42.E+08	3.20.E+08	5.57.E+07	5.07.E+09	1.55.E+08	2.23E+00	3.62E-01	
			AE-AN	1.53.E+10	4.43.E+08	1.26.E+10	5.74.E+08	6.83.E+09	6.03.E+08	1.13.E+09	1.38.E+08	8.07.E+05	1.80.E+05	1.01E-04	3.20E-05	
			AN-AN	2.45.E+09	5.97.E+08	5.95.E+08	9.85.E+07	2.24.E+09	3.62.E+08	6.90.E+08	1.01.E+08	8.83.E+05	1.53.E+05	3.02E-04	1.00E-04	
			AN-AE	1.44.E+10	2.32.E+09	1.45.E+10	1.75.E+09	5.70.E+09	1.44.E+09	7.47.E+09	1.07.E+09	3.45.E+09	2.99.E+08	2.62E-01	7.26E-02	
	Pseudomonas stutzeri	3 h	AE-AE	1.40.E+10	2.23.E+09	1.64.E+10	1.66.E+09	1.15.E+10	1.97.E+09	1.16.E+10	1.21.E+09	3.09.E+09	5.94.E+08	1.34E-01	4.41E-02	NA
			AE-AN	1.40.E+10	2.23.E+09	1.64.E+10	1.66.E+09	1.15.E+10	1.56.E+09	1.20.E+10	2.32.E+09	2.76.E+08	2.15.E+07	1.17E-02	2.85E-03	
			AN-AN	9.65.E+09	1.96.E+09	1.22.E+10	2.16.E+09	1.13.E+10	1.40.E+09	9.17.E+09	1.55.E+09	3.59E+07	5.36.E+06	1.76E-03	5.17E-04	
			AN-AE	7.35.E+09	3.79.E+08	1.00.E+10	9.52.E+08	8.57.E+09	1.36.E+09	9.03.E+09	7.51.E+08	1.21E+09	1.80.E+08	6.86E-02	1.85E-02	
		24 h	AE-AE	1.40.E+10	2.23.E+09	1.64.E+10	1.66.E+09	1.31.E+10	1.47.E+09	1.84.E+10	8.50.E+08	1.14.E+10	1.31.E+09	3.60E-01	6.79E-02	
			AE-AN	1.40.E+10	2.23.E+09	1.64.E+10	1.66.E+09	1.36.E+10	1.75.E+09	1.82.E+10	1.95.E+09	3.20.E+08	1.88.E+07	1.01E-02	1.76E-03	
			AN-AN	9.65.E+09	1.96.E+09	1.22.E+10	2.16.E+09	8.83.E+09	9.50.E+08	1.08.E+10	1.32.E+09	9.10.E+07	1.37.E+07	4.63E-03	1.23E-03	
			AN-AE	6.45.E+09	1.50.E+09	9.00.E+09	1.40.E+09	7.67.E+09	1.36.E+09	7.23.E+09	1.44.E+09	5.29.E+09	3.40.E+08	3.55E-01	8.95E-02	
Pseudomonas putida SMDBS(pCAR1::*gfp*)	*Pseudomonas stutzeri*	3 h	AE-AE	8.15.E+09	1.68.E+09	1.88.E+10	1.30.E+09	6.27.E+09	1.53.E+08	2.10.E+10	3.00.E+09	2.10.E+03	3.00.E+02	7.70E-08	1.99E-08	3.74E-03
			AE-AN	8.15.E+09	1.68.E+09	1.88.E+10	1.30.E+09	6.40.E+09	1.73.E+08	2.03.E+10	2.08.E+09	1.73.E+02	6.66.E+01	6.48E-09	3.04E-09	
		24 h	AE-AE	3.65.E+09	7.19.E+08	1.62.E+10	1.16.E+09	6.73.E+08	7.02.E+07	1.17.E+10	2.08.E+09	2.63.E+03	1.53.E+02	2.13E-07	4.96E-08	1.93E-03
			AE-AN	3.65.E+09	7.19.E+08	1.62.E+10	1.16.E+09	5.63.E+08	5.69.E+07	5.67.E+09	1.15.E+09	3.33.E+01	2.08.E+01	5.35E-09	4.38E-09	
Pseudomonas stutzeri JCM 5695(pCAR1::*gfp*)	*Pseudomonas stutzeri*	3 h	AE-AE	1.83.E+10	1.85.E+09	1.75.E+10	1.09.E+09	5.20.E+09	3.12.E+08	5.73.E+09	4.25.E+08	3.98.E+02	7.42.E+01	3.64E-08	9.24E-09	NA
			AE-AN	1.83.E+10	1.85.E+09	1.75.E+10	1.09.E+09	7.10.E+09	4.09.E+08	6.13E+09	3.01.E+08	4.73.E+02	4.31.E+01	3.58E-08	5.18E-09	
			AN-AN	1.48.E+10	1.84.E+09	8.80.E+09	7.83.E+08	5.30.E+09	7.47.E+08	4.82E+09	5.97.E+08	1.13.E+02	2.32.E+01	1.12E-08	3.78E-09	
			AN-AE	1.04.E+10	3.65.E+08	9.35.E+09	7.00.E+08	2.00.E+10	1.10.E+09	2.01E+10	1.35.E+09	3.57.E+03	5.86.E+02	8.90E-08	2.01E-08	
		24 h	AE-AE	8.20.E+09	6.32.E+08	9.00.E+09	1.36.E+09	5.10.E+08	9.54.E+07	6.30.E+08	5.29.E+07	7.93.E+03	9.45.E+02	6.96E-06	1.73E-06	
			AE-AN	8.20.E+09	6.32.E+08	9.00.E+09	1.36.E+09	8.47.E+08	1.39.E+08	1.27.E+09	1.21.E+08	3.73.E+02	4.04.E+01	1.76E-07	4.08E-08	
			AN-AN	9.10.E+09	1.44.E+09	9.05.E+09	1.01.E+09	8.70.E+08	7.55.E+07	5.17.E+08	3.79.E+07	8.00.E+01	3.61.E+01	5.77E-08	3.07E-08	
			AN-AE	7.50.E+09	1.16.E+09	1.87.E+10	5.77.E+08	1.57.E+10	9.85.E+08	1.66E+10	1.20.E+09	2.84.E+04	2.62.E+03	8.80E-07	1.41E-07	
Pseudomonas stutzeri JCM 5695(pBP136::*gfp*)	*Pseudomonas stutzeri*	3 h agar	AE-AE	1.58.E+10	1.80.E+09	1.72.E+10	2.27.E+09	9.63.E+09	1.36.E+09	9.63.E+09	8.62.E+08	1.06.E+09	1.99.E+08	5.48E-02	1.66E-02	NA
			AE-AN	1.58.E+10	1.80.E+09	1.72.E+10	2.27.E+09	7.50.E+09	9.00.E+08	1.14.E+10	8.96.E+08	7.67.E+07	5.86.E+06	4.06E-03	6.97E-04	
			AN-AN	7.45.E+09	1.37.E+09	1.30.E+10	1.21.E+09	2.83.E+09	1.90.E+08	4.90.E+09	7.00.E+08	3.43.E+06	3.90.E+05	4.44E-04	1.02E-04	
		24 h agar	AE-AE	1.48.E+10	2.57.E+09	1.49.E+10	2.68.E+09	5.13.E+09	8.62.E+08	3.21.E+09	2.37.E+08	1.66.E+10	1.14.E+09	1.99E+00	3.98E-01	
			AE-AN	1.48.E+10	2.57.E+09	1.49.E+10	2.68.E+09	7.10.E+09	6.56.E+08	5.57.E+09	9.07.E+08	1.05.E+08	1.18.E+07	8.29E-03	1.95E-03	
			AN-AN	9.25.E+09	1.32.E+09	1.21.E+10	1.74.E+09	5.33.E+09	8.50.E+08	5.17.E+09	9.61.E+08	8.93.E+06	1.31.E+06	8.51E-04	2.71E-04	

Table S1-2. *P* values of multiple-comparisons by Tukey HSD test for data in Figures 3 and S2.

Figure 3A / Duration	Condition	AE-AE	AE-AN	AN-AN	AN-AE
3 h	AE-AE	-	-	-	-
	AE-AN	9.0E-04	-	-	-
	AN-AN	1.7E-03	9.3E-01	-	-
	AN-AE	5.8E-01	3.9E-03	8.1E-03	-
24 h	AE-AE	-	-	-	-
	AE-AN	3.5E-04	-	-	-
	AN-AN	3.4E-04	1.0E+00	-	-
	AN-AE	1.0E+00	4.3E-04	4.2E-04	-

Figure 3B / Duration	Condition	AE-AE	AE-AN	AN-AN	AN-AE
3 h	AE-AE	-	-	-	-
	AE-AN	6.7E-05	-	-	-
	AN-AN	6.4E-05	1.0E+00	-	-
	AN-AE	8.0E-04	9.3E-02	8.8E-02	-
24 h	AE-AE	-	-	-	-
	AE-AN	2.1E-06	-	-	-
	AN-AN	2.1E-06	1.0E+00	-	-
	AN-AE	5.3E-06	3.7E-01	3.7E-01	-

Figure 3C / Duration	Condition	AE-AE	AE-AN	AN-AN	AN-AE
3 h	AE-AE	-	-	-	-
	AE-AN	1.1E-03	-	-	-
	AN-AN	6.5E-04	9.5E-01	-	-
	AN-AE	4.1E-02	7.6E-02	3.7E-02	-
24 h	AE-AE	-	-	-	-
	AE-AN	2.8E-04	-	-	-
	AN-AN	2.5E-04	1.0E+00	-	-
	AN-AE	1.0E+00	3.1E-04	2.8E-04	-

Figure 3D / Duration	Condition	AE-AE	AE-AN	AN-AN	AN-AE
3 h	AE-AE	-	-	-	-
	AE-AN	1.0E+00	-	-	-
	AN-AN	1.0E-01	1.1E-01	-	-
	AN-AE	2.3E-03	2.1E-03	1.6E-04	-
24 h	AE-AE	-	-	-	-
	AE-AN	5.5E-05	-	-	-
	AN-AN	4.8E-05	1.0E+00	-	-
	AN-AE	1.2E-04	7.6E-01	6.7E-01	-

Figure S2 / Duration	Condition	AE-AE	AE-AN	AN-AN	AN-AE
3 h	AE-AE	-	-	-	-
	AE-AN	1.6E-03	-	-	-
	AN-AN	1.1E-03	8.9E-01	-	-
24 h	AE-AE	-	-	-	-
	AE-AN	1.0E-04	-	-	-
	AN-AN	1.0E-04	1.0E+00	-	-

Table S1-3. Transconjugant frequency of plasmids under different conditions of oxygen from *Pseudomonas putida* to fucaltative anaerobes.

plasmid	mating duration (h)	condition	*Buttiauxella agrestis*			*Pantoea agglomerans*			*Pseudomonas stutzeri*		
			Ave	Stdev	*p* value	Ave	Stdev	*p* value	Ave	Stdev	*p* value
pBP136	3	AE_AE	1.1E-06	2.5E-07	4.9E-01	4.3E-04	7.2E-05	8.1E-05	4.1E-04	1.2E-04	1.5E-03
	24	AE_AE	1.3E-06	4.0E-07		1.2E-01	1.2E-02		2.9E-02	6.4E-03	
	3	AE_AN	4.0E-07	1.1E-07	2.0E-01	3.0E-05	3.7E-06	4.8E-02	5.3E-05	8.6E-06	1.0E-02
	24	AE_AN	6.2E-07	2.3E-07		2.4E-05	1.9E-06		2.9E-04	9.1E-05	
pCAR1	3	AE_AE	-	-	-	-	-	-	7.7E-08	2.0E-08	3.7E-03
	24	AE_AE	-	-	-	-	-	-	6.5E-09	3.0E-09	
	3	AE_AN	-	-	-	-	-	-	2.1E-07	5.0E-08	1.9E-03
	24	AE_AN	-	-	-	-	-	-	5.4E-09	4.4E-09	

Table S1-4. Transconjugant frequency of plasmids under different conditions of oxygen from *Pseudomonas stutzeri* to fucaltative anaerobes.

plasmid	mating duration (h)	condition	*Buttiauxella agrestis*			*Pantoea agglomerans*			*Pseudomonas stutzeri*		
			Ave	Stdev	*p* value	Ave	Stdev	*p* value	Ave	Stdev	*p* value
pBP136 (Fig. 2ABC)	3	AE_AE	2.8E-05	5.7E-06	3.7E-03	1.3E-02	3.3E-03	4.4E-04	1.3E-01	4.4E-02	8.4E-03
	24	AE_AE	6.9E-04	1.9E-04		2.2E+00	3.6E-01		3.6E-01	6.8E-02	
	3	AE_AN	1.3E-06	2.6E-07	2.7E-02	9.8E-05	1.9E-05	9.0E-01	1.2E-02	2.8E-03	4.4E-01
	24	AE_AN	3.7E-06	1.2E-06		1.0E-04	3.2E-05		1.0E-02	1.8E-03	
	3	AN_AN	3.8E-06	7.4E-07	1.5E-02	3.5E-05	9.2E-06	1.0E-02	1.8E-03	5.2E-04	2.1E-02
	24	AN_AN	1.6E-06	5.4E-07		3.0E-04	1.0E-04		4.6E-03	1.2E-03	
	3	AN_AE	2.3E-05	8.3E-06	9.2E-04	3.9E-03	8.3E-04	3.5E-03	6.9E-02	1.8E-02	5.6E-03
	24	AN_AE	6.7E-04	1.3E-04		2.6E-01	7.3E-02		3.6E-01	8.9E-02	
pBP136 (Fig. S2)	3	AE_AE	-	-	-	-	-	-	5.5E-02	1.7E-02	1.09E-03
	24	AE_AE	-	-	-	-	-	-	2.0E+00	4.0E-01	
	3	AE_AN	-	-	-	-	-	-	4.1E-03	7.0E-04	2.43E-02
	24	AE_AN	-	-	-	-	-	-	8.3E-03	2.0E-03	
	3	AN_AN	-	-	-	-	-	-	4.4E-04	1.0E-04	7.17E-02
	24	AN_AN	-	-	-	-	-	-	8.5E-04	2.7E-04	
pCAR1 (Fig. 2D)	3	AE_AE	-	-	-	-	-	-	3.6E-08	9.2E-09	2.3E-03
	24	AE_AE	-	-	-	-	-	-	7.0E-06	1.7E-06	
	3	AE_AN	-	-	-	-	-	-	3.6E-08	5.2E-09	4.1E-03
	24	AE_AN	-	-	-	-	-	-	1.8E-07	4.1E-08	
	3	AN_AN	-	-	-	-	-	-	1.1E-08	3.8E-09	6.0E-02
	24	AN_AN	-	-	-	-	-	-	5.8E-08	3.1E-08	
	3	AN_AE	-	-	-	-	-	-	8.9E-08	2.0E-08	6.5E-04
	24	AN_AE	-	-	-	-	-	-	8.8E-07	1.4E-07	

Table S2. Genera of transconjugants obtained by mating with microbes from environmental samples under aerobic (AE) and anaerobic (AN) conditions with another cow manure sample (#2) with mating on LB or Agar plate.

Top hit[a]	Total		cow manure #2 (LB)		cow manure #2 (Agar)		Family	Order	Class	Phylum
(Genus level)	AE	AN	AE	AN	AE	AN				
Candidimonas	**1**	**0**	0	0	1	0	*Alcaligenaceae*	*Burkholderiales*	*Beta-proteobacteria*	*Proteobacteria*
Pusillimonas	**1**	**0**	0	0	1	0				
Escherichia	**31**	**42**	8	2	23	40	*Enterobacteriaceae*	*Enterobacteriales*	*Gammaproteobacteria*	
Klebsiella	**2**	**0**	0	0	2	0				
Raoultella	**3**	**0**	0	0	3	0				
Shigella	**34**	**57**	15	22	19	35				
Pseudomonas	**27**	**0**	0	0	27	0	*Pseudomonadaceae*	*Pseudomonadales*		
Stenotrophomonas	**1**	**0**	0	0	1	0	*Xanthomonadaceae*	*Xanthomonadales*		
Total	**100**	**99**	**23**	**24**	**77**	**75**				

[a]Genera of transconjugants that showed >97% identity in the BLAST search with EzTaxon server 2.1 (23, www.eztaxon.org/). Genera uniquely obtained under aerobic (AE) conditions are shown in red.