Divalent cations increase the conjugation efficiency of the incompatibility P-7 group plasmid pCAR1 among different Pseudomonas hosts

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Abstract

The incompatibility (Inc) P-7 group plasmid pCAR1 can be efficiently transferred among bacteria in artificial microcosms in the presence of divalent cations Ca²⁺ and Mg²⁺. One-on-one mating assays between Pseudomonas strains with different plasmids showed that the promotion of conjugation efficiency by divalent cations was exhibited in other plasmids, including pB10 and NAH7; however, this effect was larger in IncP-7 plasmids. The impact on pCAR1 conjugation differed according to donor–recipient pairs, and conjugation efficiency promotion was clearly detected between the donors P. resinovorans CA10dm4 and P. fluorescens P70-1 and the recipients P. putida KT2440 and CA10dm4. Transcriptome analyses showed that pCAR1 gene expression did not respond to cation changes, including the tra/trh genes involved in its transfer. However, the transcription of oprH genes, encoding putative outer-membrane proteins in both the donor and the recipient, were commonly upregulated under cation-limited conditions. The conjugation frequency of pCAR1 in the KT2440 oprH mutant was found not to respond to cations. This effect was partially recovered by complementation with the oprH gene, suggesting that OprH is involved in the increase of pCAR1 conjugation efficiency by divalent cations.

INTRODUCTION

Conjugative plasmids transfer various genes and endow their host recipients with new phenotypes, including antibiotic and heavy metal resistance, pathogenicity and metabolism. Therefore, their transfer is an important phenomenon promoting rapid bacterial evolution and adaptation in various environments. The frequency of plasmid conjugation is affected by many factors, including the type of sex pili (rigid or flexible), host surroundings (solid surface or liquid environment), sex pheromones produced by gene products on some plasmids, cell density, growth rate, host species, nutrients, temperature and high-salt stress [1–8]. The identification of these factors and the mechanisms that determine how they affect conjugation frequency is important for understanding and predicting plasmid behaviour in diverse environments.

The incompatibility (Inc) P-7 group plasmid pCAR1 was discovered in Pseudomonas resinovorans CA10 as a carbazole-degradative plasmid [9–12]. pCAR1 is a self-transmissible plasmid that is mainly found among genus Pseudomonas bacteria [3, 11]. Studies of pCAR1 transfer in model artificial microcosms have shown that this plasmid preferably transfers in aqueous environments [13–15], while composition analysis showed that the presence of the divalent cations Ca²⁺ and Mg²⁺ increased the conjugation efficiency of this plasmid [13]. These cations are known to be important for cell-to-cell attachment in Pseudomonas [16–19] and a recent review argued that they are also important in DNA synthesis [20]; however, their effects on the conjugation of plasmids remain unknown. The objective of this study was to understand the effects of cations on plasmid conjugation as an environmental factor. We assessed whether the presence of exogenous cations could influence the transferability of other plasmids, and whether the effects of cations on plasmids differed according to donor and recipient Pseudomonas cells. We used transcriptome
comparisons to explore the ability of a host or plasmid factors to respond to the presence of exogenous cations during plasmid conjugation.

METHODS

Bacterial strains, media, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. We grew Escherichia coli JM109 at 37 °C in lysogeny broth (LB) for cloning, whereas Pseudomonas strains were grown at 30 °C in two-fold diluted LB (1/2LB). Ampicillin (Ap, 50 µg ml⁻¹), chloramphenicol (Cm, 30 µg ml⁻¹), gentamicin (Gm, 30 µg ml⁻¹), kanamycin (Km, 50 µg ml⁻¹), rifampicin (Rif, 25 µg ml⁻¹) and tetracycline (Tc, 12.5 µg ml⁻¹) were added to provide selective medium. The media were solidified using 1.6 % (wt vol⁻¹) purified agar powder (Nacalai Tesque, Kyoto, Japan) for plate cultures.

To assess the effects of divalent cations on plasmid conjugation frequency, P. putida SM1443, a derivative strain of KT2440, was used as a donor, and P. putida KT2440RG was used as a recipient for pCAR1::rfp, pB10::rfp, R388::rfp and NAH7K2 (Table 1). For pDK1K, P. fluorescens Pf-5 derivative strains were used as donor and recipient strains (Table 1). Mating assays to test the effects of cations were performed using four donors [SM1443(pCAR1::rfp), CA10L(pCAR1::rfp), P. fluorescens Pf-5(pCAR1::rfp) and JCM 2778L(pCAR1::rfp)] and five recipients (KT2440RG, CA10dm4RG, P. putida SM1443(pCAR1::rfp), and CA10dm4RG, JCM 13063RG and JCM 2778RG) (Table 1). All of the experiments were performed at least twice. Overnight cultures of donor and recipient cells in 1/2LB were harvested by centrifugation and washed with 1 ml carbon-free (CF) buffer [2.2 g Na2HPO4, 0.8 g KH2PO4 and 3.0 g NH4NO3 (Kanto Chemical) in 1 l distilled and ion-exchanged water] five times.

One-on-one mating experiments

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
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<tr>
<td>Escherichia coli JM109</td>
<td>F’ [traD36, proAB, lacI, lacZAM15], recA1, endA1, gyrA96, thi-1, hsdR17(k8, m26), c14 (merA), supE44, relA1, Δ(lac-proAB)</td>
<td>RBCBioscience</td>
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<td>JCM 2778RG</td>
<td>Derivative strain of JCM 2778 (previous name, IAM 1511), spontaneously Rif’, with Gm’ gene inserted into chromosome</td>
<td>[3]</td>
</tr>
<tr>
<td>JCM 2778L(pCAR1::rfp)</td>
<td>Derivative strain of JCM 2778 with lacI’ gene inserted into chromosome bearing pCAR1::rfp</td>
<td>[14]</td>
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<td><strong>Pseudomonas fluorescens</strong></td>
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<td></td>
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<td>P0-1RG</td>
<td>Derivative strain of P0-1, spontaneously Rif’ with Gm’ gene inserted into chromosome</td>
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<td>P0-1L bearing pCAR1::rfp</td>
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<td>Derivative strain of KT2440, Tn5 was inserted into 1 360 499 nt of chromosome</td>
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<td>KT2440 bearing pCAR1omrHis with Gm’ gene cassette and FRT sites</td>
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<td><strong>Plasmids</strong></td>
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<td>pBBR1MCS-3</td>
<td>Tc’, lacZa mob, compatible with IncP, IncQ and IncW plasmids</td>
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<td>pBBRoprH</td>
<td>pcBBR1MCS-3, oprH (PP_1185, 1360133–1360738 nt of KT2440) at the SfuI site of the vector under the lac promoter by the Gibson assembly system (the SfuI site was no longer available)</td>
<td>This study</td>
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The cells were suspended in CF buffer that was equivalent to 0.2 turbidity at 600 nm (OD₆₀₀) in the case of donors and suspended in CF buffer that was equivalent to 2.0 turbidity in the case of recipients. Equal volumes (200 µl) of donor and recipient cells were mixed in 2 ml microtubes with and without exogenous cations (CaCl₂ and MgSO₄) at 0.4, 4, 40 and 400 µM. The microtube caps were left open and the lids were sealed with gas-permeable adhesive seals (Thermo Fisher Scientific). After incubation for 24 h at 30 °C, triplicate aliquots (10 µl) of serial-diluted cultures were spotted onto selective plates. The conjugation frequencies were calculated as the ratio of transconjugants [colony-forming units (c.f.u.) ml⁻¹]/donors (c.f.u. ml⁻¹).

For the transcriptome analysis of the donor [PF₀-1L (pCAR₁ :: rfp): PF] and recipient (KT2440RG: KT), the overnight cell cultures of the donor and the recipient were suspended in 4 ml of CF buffer and adjusted to a turbidity of 2.0. Each 4 ml suspension was then mixed in a 50-ml tube [PF+KT]. For the single donor [PF] and recipient [KT] samples, 4 ml of each culture was mixed with 4 ml of CF buffer in a 50-ml tube. The concentration of the exogenous cations was 0 µM [cation minus (CM)] or 400 µM [cation plus (CP)], respectively. The mating duration was 24 h, after which the donor, recipient, and transconjugant c.f.u.s were counted using 200 µl aliquots to confirm the effect of cations on the conjugation of pCAR₁ :: rfp.

In the mating assays of the donor strain with mutant recipients, pCAR₁ pmrHis :: Gm [22] was transferred from KT2440(pCAR₁ pmrHis :: Gm) to PF₀-1L.

Standard DNA manipulation

Standard methods were used in the extraction of plasmid DNA and DNA digestion using restriction endonucleases [21]. Competent E. coli JM109 (RBCBioscience) cells were used for transformation. The primers used in this study are listed in Table S1, available in the online version of this article. Total DNA was extracted from Pseudomonas strains using hexadecyltrimethylammonium bromide [23]. Electroporation of Pseudomonas was performed according to the method described by Itoh et al. [24].

RNA extraction

RNA extraction from Pseudomonas strains was performed as described previously [25]. Briefly, bacterial cells were treated with RNAProtect bacteria reagent (Qiagen) to stabilize the RNA before extraction. Total RNAs were then extracted using Nucleospin RNA II (Macherey-Nagel). The eluted RNAs were treated with RQ1 RNase-free DNase (Promega) and then purified again on a Nucleospin RNA binding column (Macherey-Nagel).

Tiling array transcriptome analyses of pCAR₁ and the PF₀-1 and KT2440 chromosomes

Transcriptome analyses of P. fluorescens PF₀-1L(pCAR₁ :: rfp) and P. putida KT2440RG were performed using total RNA from the donor [PF], the recipient [KT] and a mixture of these [PF+KT]. Total RNA was extracted from biologically duplicated samples of 7.8 ml [PF+KT] or 8 ml ([PF] and [KT]) following 24 h incubation in CF buffer in the absence and presence of exogenous cations. Analyses with custom-made tiling arrays were performed as described previously [25–27]. Briefly, total RNA was extracted and single-stranded cDNA was synthesized in the presence of actinomycin D (Sigma-Aldrich) to prevent the generation of spurious second-strand cDNA. After the fragmentation and labelling of the cDNA, 5 µg of labelled cDNA was hybridized individually with each custom tiling chip. After the intensities for each probe were computed, the median signal intensities of the probes covering the region from the start to the stop codon were defined as the transcriptional values for each gene. As discussed previously [27], we defined transcription values greater than 64 as showing significant transcriptional activity. Comparisons between samples were performed using all of the biologically duplicated data, and we identified up- and downregulated open reading frames with fold changes exceeding 2.0 in all four data comparisons (between replicate 1 or 2 of sample A and replicate 1 or 2 of sample B). The data were visualized using the IGB software package (Affymetrix).

Construction of the plasmid for a complementation assay of the oprH gene

The DNA region containing oprH was amplified by polymerase chain reaction (PCR) using PrimeSTAR GXL (TAKARA BIO), with the primers listed in Table S1. The resultant fragment was cloned into a broad host range vector pBBR1MCS-3 [28] using the Gibson assembly system (New England Biolab), yielding pBBRoprH. The nucleotide sequences of the insert were confirmed by Sanger sequencing (Eurofins Genomics, Japan). The resultant plasmids were introduced into KT2440ΔoprH by electroporation.

Statistical analyses

The data to assess the effect of cations on the conjugation frequency of different plasmids were investigated using the F-test and Student’s t-tests (P<0.05). Differences in conjugation frequencies from the donor PF₀-1L(pCAR₁ pmrHis :: Gm) to KT2440KR, the ΔoprH-mutant (KT2440ΔoprH) and KT2440ΔoprH with pBBRoprH were analysed using Bartlett’s test and the Kruskal–Wallis test (P<0.05). Multiple comparisons of these data were performed using the Steel–Dwass test (P<0.05).

Microarray data accession number

The array data reported in this article were deposited in the Gene Expression Omnibus of the National Center for Biotechnology Information (NCBI) (GEO; http://www.ncbi.nlm.nih.gov/geo/) under the GEO series accession no. GSE97565.
RESULTS AND DISCUSSION

The presence of exogenous cations increased the conjugation frequency of plasmids

We performed comparisons of conjugation frequency by one-on-one mating assays using a KT2440-derivative donor (SM1443) and recipient strains with pCAR1 :: rfp (IncP-7), pB10 :: rfp (IncP-1), NAH7K2 (IncP-9) and R388 :: rfp (IncW) in the presence or absence of exogenous Ca\(^{2+}\) and Mg\(^{2+}\). pCAR1 :: rfp, pB10 :: rfp and NAH7K2 were found to show statistically significantly higher conjugation frequencies in the presence of exogenous Ca\(^{2+}\) or Mg\(^{2+}\) compared to media without exogenous cations (P<0.05; Fig. 1a–c). The conjugation of R388 :: rfp was not affected by the cations (P>0.05; Fig. 1d). The difference in conjugation frequency between cases where exogenous cations were present and cases where they were absent was the greatest for pCAR1 :: rfp. We also assessed the effect of these cations on another IncP-7 plasmid, pDK1 (pDK1K). Because pDK1 is not hosted by P. putida KT2440 [29], P. fluorescens Pf-5-derivative strains were used as the donor and recipient strains. Its conjugation frequency was also significantly higher in the presence of the exogenous cations (P<0.05) (Fig. 1e). These results showed that the presence of exogenous cations in mating assays increased plasmid conjugation frequency, and that the effect was greater in the case of IncP-7 plasmids. Therefore, subsequent in-depth analyses of the effects of these divalent cations on plasmid conjugation were performed using pCAR1.

The effects of exogenous cations and their concentrations on pCAR1 :: rfp conjugation efficiency were altered according to donor–recipient mating pairs

We performed one-on-one mating assays in the presence and absence of exogenous cations using different donor–recipient pairs. We used four donor strains [P. putida SM1443(pCAR1 :: rfp), P. resinovorans CA10L(pCAR1 :: rfp), P. fluorescens Pf-0-1L(pCAR1 :: rfp) and P. chlororaphis subsp. chlororaphis JCM 2778L(pCAR1 :: rfp)] and five recipient strains (P. putida KT2440RG, P. resinovorans CA10dm4RG, P. fluorescens Pf0-1RG, P. putida JCM 13063RG and P. chlororaphis subsp. chlororaphis JCM 2778RG). Before the assays, the transferability of pCAR1 :: rfp was confirmed by mating assays on nutrient-rich media (1/2LB) in all possible donor–recipient pairs (data not shown). The range of exogenous Ca\(^{2+}\) and/or Mg\(^{2+}\) concentrations was 0–400 µM.

The presence of exogenous Ca\(^{2+}\) and Mg\(^{2+}\) cations was found to affect the conjugation frequency of pCAR1 :: rfp from SM1443(pCAR1 :: rfp) to KT2440RG, CA10dm4RG and Pf0-1RG, and the frequency rose as their concentrations increased (Fig. 2a–c), whereas the conjugation frequencies to JCM 13063RG and JCM 2778RG were not affected by the presence of exogenous cations (Fig. 2d, e; for the statistical analyses, see Supplemental Text S1 and Table S2). Similarly, significant differences in the conjugation frequency of the pCAR1 :: rfp between lower and higher concentration of these cations were detected between CA10L(pCAR1 :: rfp) and KT2440RG, CA10dm4RG, or Pf0-1RG, and between Pf0-1L(pCAR1 :: rfp) and KT2440RG and CA10dm4RG (Fig. S1a–c, f, and g, Supplemental Text S1, Table S2). However, the conjugation frequency was below the detection limit (<10\(^{-7}\) per donor) for some donor–recipient pairs (Fig. S1d, e, h and j).

When JCM 2778L(pCAR1 :: rfp) was used as a donor, plasmid conjugation was detected at low frequencies; however, no effects were detected for cations (Fig. S1k–o, Supplemental Text S1, Table S2). As previously reported, IncP-7 transfer is restricted by the conjugation system rather than by the replication system [29], and JCM 2778 was found to be a poor donor or recipient for pCAR1 conjugation.

![Fig. 1. Conjugation frequency of plasmids in the presence or absence of exogenous divalent cations (400 µM Ca\(^{2+}\) and Mg\(^{2+}\)). Mating assays were performed between (a)–(d) Pseudomonas putida SM1443 (donor) and P. putida KT2440RG (recipient), and between (e) P. fluorescens Pf-55 (donor) and P. fluorescens Pf-5G (recipient). (a) pCAR1 :: rfp (IncP-7), (b) pB10 :: rfp (IncP-1), (c) NAH7K2 (IncP-9), (d) R388 :: rfp (IncW) and (e) pDK1K (IncP-7). The means of the conjugation frequency (transconjugant/donor) in experiments performed three (a)–(d) and five times (e) are shown as bars. Filled diamonds indicate the frequencies of each experiment. Asterisks indicate significant differences between two conditions as assessed by the F-test and Student’s t-test (P<0.05) (n=3 for pCAR1 :: rfp, pB10 :: rfp, NAH7K2 and R388 :: rfp, and n=5 for pDK1K).]
In summary, the clearest impacts of the exogenous Ca$^{2+}$ and Mg$^{2+}$ cations on pCAR1::rfp conjugation were found between CA10L(pCAR1::rfp) or Pf0-1L(pCAR1::rfp) donors and KT2440RG or CA10dm4RG recipients, and the conjugation frequency of the plasmid between these strains in the absence of exogenous cations was below the detection limit (Fig. S1a, b, f and g).

**Transcriptome comparisons in the presence or absence of exogenous divalent cations**

To understand how exogenous Ca$^{2+}$ and Mg$^{2+}$ cations increased pCAR1 conjugation efficiency, we performed transcriptome comparisons of donor and recipient cells in the absence and presence of exogenous cations. Pf0-1L(pCAR1::rfp) and KT2440RG were used as the donors and recipients because the impact of exogenous cations on this pair was larger than that on other pairs (Fig. S1f), and because our custom tiling arrays were available for the Pf0-1L(pCAR1::rfp) donor and KT2440RG recipient.

Given that cations can affect the adhesion of bacterial cells [16–19], we performed microscopic observation and no physical adhesions between donor and recipient cells were detected in the presence of exogenous cations (Supplemental Text S1, Fig. S2). In addition, we confirmed that these cations did not affect the growth or stability of the plasmid in the donor and transconjugants (Supplemental Text S1, Fig. S3).

To assess which genes were responsible for increasing the conjugation frequency of pCAR1 in response to the cations, we compared transcriptomes of the donor [Pf0-1L(pCAR1::rfp), PF], the recipient (KT2440RG, KT) and a mixture of the donor and the recipient [PF+KT] in the absence [0 µM Ca$^{2+}$ and Mg$^{2+}$, cation minus (CM)] and presence [400 µM Ca$^{2+}$ and Mg$^{2+}$, cation plus (CP)] of exogenous cations. Although the donor cell density was 10-fold higher than that in the experiments shown in Fig. S1f, a similar effect due to these cations was found in the mating assay (data not shown).

The transcriptome of the genes on pCAR1 in the donor [PF] and mixture [PF+KT] were compared in the presence and absence of exogenous cations. However, the genes on pCAR1, including the *tra*/*trh* genes involved in its conjugation, were not differentially transcribed in either case (Table S3-1). This result suggests two possibilities: (i) the cations might not strongly promote the transcription of the genes on pCAR1, including the *tra*/*trh* genes, or (ii) the differences in gene transcription on pCAR1 could not be detected because the
cations might not increase plasmid conjugation efficiency in every donor cell. It is also possible that cations affect gene expression on chromosomal copy of the donor, the recipient, or both, rather than only those on the plasmid.

The transcriptomes of genes on donor and/or recipient chromosomes were compared in the presence and absence of the exogenous cations (Tables S3-2 and S3-3). Eleven genes in the donor [PF] sample and 123 genes in the recipient [KT] sample were found to be differentially transcribed in the presence of exogenous cations (Fig. 3 and Tables S4-1 and S4-3). In the mixtures [PF+KT], 7 genes were found in the donor and 126 genes were found in the recipient (Fig. 3, Tables S4-2 and S4-4). Among these, 31 recipient genes were commonly downregulated in both the [KT] and the [PF+KT] samples (Fig. 3b), whereas no donor genes were commonly regulated in the [PF] or [PF+KT] samples (Fig. 3a).

In the presence of exogenous cations, the conjugation frequency of pCAR1::rfp increased – not only from Pfo-1L (pCAR1::rfp) to KT2440RG (Fig. S1f), but also from SM1443(pCAR1::rfp) to Pfo-1RG (Fig. 2c; note that SM1443 is a derivative strain of KT2440). These results imply that the genes that are putatively responsive to the exogenous cations could be in either the donor or the recipient chromosome, or in both. Thus, we targeted the orthologous genes between KT2440 and Pfo-1, and selected similarly responding genes from the above 11 and 7 genes in the donor (Fig. 3a) and the 123 and 126 genes in the recipient (Fig. 3b). As a result, 7/11 genes and 5/7 donor genes had orthologous genes on the recipient, and only PP_1185 was selected as a commonly downregulated gene in [KT] and [PF+KT], whose orthologous gene Pfo01_4241 was found in [PF+KT] (Tables S4-2, S4-3 and S4-4). Although other differentially transcribed genes may have been involved in the increase of pCAR1 conjugation efficiency by the exogenous cations, we focused on Pfo01_4241 and PP_1185 as commonly differentially transcribed genes.

**Effects of cations on pCAR1 conjugation efficiency with oprH (PP_1185) disruptant of KT2440**

Pfo01_4241 and PP_1185 are predicted to encode outer-membrane protein H1 (OprH), although their function remains unclear. To assess whether the products of oprH (PP_1185) could be involved in the increase of pCAR1 conjugation efficiency in the presence of the exogenous cations, we performed mating assays with the oprH disruptant of KT2440 (KT2440ΔoprH) and a complementation assay in KT2440ΔoprH with pBBRoprH. For the selection of transconjugants, we used Pfo0-1L(pCAR1pmrHis::Gm) and KT2440KR instead of Pfo0-1L(pCAR1::rfp) and KT2440RG. Before the assays, the effect of Ca2+ and Mg2+ cations was confirmed in these donors and recipients [wild-type (WT); Fig. 4], and the transferability of the plasmid from the donor to KT2440ΔoprH was confirmed in nutrient-rich conditions (data not shown). The conjugation frequency of plasmid to KT2440ΔoprH in the presence of these exogenous cations was as low as that in their absence (Fig. 4). The conjugation frequency of pCAR1pmrHis::Gm to the complemented strain was as high as that in the absence (Fig. 4). The conjugation frequency of pCAR1pmrHis::Gm to the complemented strain was statistically significantly higher than that in their absence (P<0.05), although the frequency was not as high as that in the WT (Fig. 4). These results indicated that OprH was not an essential protein for pCAR1 conjugation but might be required for its efficient conjugation by cooperation with the exogenous cations, although its expression was repressed under that condition. The expression level of OprH might be important for the response to these cations. A possible explanation for the fact that the conjugation frequency of the complemented strain was not as high as that of the WT in the presence of exogenous cations is the possibility that the expression level of the oprH in pBBRoprH might be either too low or too high in comparison to that of the WT.

In-depth analyses of the expression levels of oprH genes will be required in these strains (WT, KT2440ΔoprH and the complemented strain) in future work.

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**Fig. 3.** Venn diagram of differentially transcribed coding sequences in the donor strain Pfo0-1L(pCAR1::rfp) chromosome (a) and the recipient strain KT2440RG chromosome (b). The presence and absence of exogenous cations are labelled as 'CM' (cation minus). The number of differentially transcribed donor genes in the donor sample [PF] and the sample containing a mixture of donor and recipient [PF+KT] (a), and the number in the recipient sample [KT] and [PF+KT] (b) in the absence and presence of cations is shown.
OprH was found in *P. aeruginosa* PAO1 as an important outer-membrane protein involved in resistance to cationic antibiotics under Mg\(^{2+}\)-limited conditions linked with the two regulatory system components, PhoP and PhoQ [30–32], and was also predicted to have an accessory role in membrane stabilization [33]. Recently, interaction between OprH and lipopolysaccharides (LPS) was reported in PAO1 [34, 35]. OprH was thought to function instead of the divalent cations in the LPS for the stabilization of the outer membrane in PAO1 [34]. The function of OprH in KT2440 is unclear, although it has been found in outer-membrane vesicles [36]. Our results indicate that both KT2440 OprH and cations are involved in efficient plasmid conjugation (Fig. 4). The stability of the outer membrane might be an important factor in plasmid conjugation. It should be noted that oprH homologous genes were conserved in the four *Pseudomonas* strains used in this study whose genome sequences were available (KT2440, CA10dm4, Pf0-1 and JCM 13063). Nevertheless, the effects of exogenous Ca\(^{2+}\) and Mg\(^{2+}\) cations on plasmid conjugation efficiency differed between donor–recipient pairs. It will be also necessary to compare their expression levels and ratios in the outer membranes.

The fact that the complemented strain showed only a partial recovery of the response to the cations also suggests that other gene products could influence the conjugation efficiency. In KT2440, another outer-membrane protein-encoding gene, oprQ, was listed as a downregulated gene in the presence of cations in the mixture (Tables S4–4). The oprQ gene has been found in *P. aeruginosa* [37], but its function is also unknown. It is possible that changes in the ratio of these outer-membrane proteins in the cell membrane affect plasmid conjugation.

### Conclusion

Divalent cations are important factors in various activities of cells, enzymes and chemical reactions. The IncP-7 plasmid pCAR1 was efficiently transmitted in the presence of cations in several donor–recipient pairs of *Pseudomonas* strains. Transcriptome analyses and mating assays with a mutant and complemented strain showed that a host factor (OprH) was involved in the conjugation efficiency of pCAR1, together with an environmental factor (cations). Our findings are important for the understanding of plasmid behaviours in different environments. However, we still do not have an in-depth understanding of the related molecular mechanisms. It will be necessary to assess the stability of the outer membranes of donor and recipient cells in the presence and absence of cations. It is also reported that cations influence the cell surface conditions of *Pseudomonas aeruginosa* PAO1 [38]. Other factors may increase plasmid conjugation efficiency in the presence of cations. Identifying such factors by transcriptome analyses is difficult because conjugation events promoted by the cations occurred approximately once in 10\(^8\) cells in the donor (Fig. S1F). Our transcriptome analyses were performed using total RNA extracted from 10\(^6\) to 10\(^7\) cells; therefore, although there were donor cells with differentially transcribed genes in response to the presence of cations, they may have constituted less than 0.01% of the population and remained undetected. Indeed, the genes involved in pCAR1 conjugation efficiency were not found to be highly transcribed in our previous studies [26, 39]. This may be because we did not detect any genes on pCAR1 that were differentially transcribed in the presence or absence of exogenous cations (Table S3-1). To identify the factors increasing plasmid conjugation efficiency in the presence of cations, it will be necessary to separate and analyse the single cells that transfer plasmid in the presence of cations.

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### Conflicts of interest

The authors declare that there are no conflicts of interest.

### References


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