JB Accepts, published online ahead of print on 7 February 2014
J. Bacteriol. doi:10.1128/JB.01464-13

Plasmid conjugation from Proteobacteria as evidence for the origin of xenologous genes in Cyanobacteria

David Encinas[a*], M. Pilar Garcillán-Barcia[a*], María Santos-Merino[a], Luis Delaye[b], Andrés Moya[c] and Fernando de la Cruz[a#]

Instituto de Biomedicina y Biotecnología de Cantabria IBBTEC (CSIC–Universidad de Cantabria-SODERCAN)[a]; Departamento de Ingeniería Genética CINVESTAV-Irapuato, Irapuato, Guanajuato, México[b]; Institut Cavanilles de Biodiversitat i Biologia Evolutiva, Universitat de Valencia, Valencia, Spain[c]

Running Title: Plasmid conjugation to *Synechococcus*

* DE and MPGB equally contributed to this work

[#]Address correspondence to F. de la Cruz, delacruz@unican.es

Keywords: Cyanobacteria, plasmid conjugation, type IV secretion system, synthetic biology, shuttle vector, horizontal gene transfer, Synechococcus elongatus PCC 7942

Abstract

Comparative genomics showed that 5% of *Synechococcus elongatus* PCC 7942 genes are of probable proteobacterial origin. To investigate the role of inter-phylum conjugation in cyanobacterial gene acquisition, we tested the ability of a set of prototype proteobacterial conjugative plasmids (RP4, pKM101, R388, R64 and F) to transfer DNA from *E. coli* to *S. elongatus*. A series of BioBrick-compatible, mobilizable shuttle vectors was developed. These vectors were based on the putative origin of replication of the *Synechococcus* resident plasmid pANL. Not only broad-host range plasmids, such as RP4 and R388, but also narrower host-range plasmids, such as pKM101, all encoding MPF_T-type IV secretion systems, were able to transfer plasmid DNA from *E. coli* to *S. elongatus* by conjugation. Neither MPF_F, nor MPF_I could be used as interphylum DNA delivery agents. Reciprocally, pANL-derived cointegrates could be introduced in *E. coli* by electroporation, where they conferred a functional phenotype. These results suggest the existence of potentially ample channels of gene flow between Proteobacteria and Cyanobacteria and point to MPF_T-based inter-phylum conjugation as a potential mechanism to explain the proteobacterial origin of a majority of *S. elongatus* xenologous genes.

36 ***Introduction***

37

38 Horizontal gene transfer (HGT) is an outstanding player of bacterial evolution (1).
39 Among classical HGT mechanisms, natural transformation was demonstrated in several
40 cyanobacteria, including *Synechococcus elongatus* PCC 7942 (hereinafter Se7942) (2),
41 *Synechococcus* sp. PCC 7002 (3) and *Synechocystis* sp. PCC 6803 (4), while conjugative
42 transfer among *Anabaena* strains was also reported (5). Although no experimental
43 evidence for transduction has been reported, several marine phages that contain
44 photosynthetic genes have been detected (6, 7). This fact could indicate that
45 photosynthetic genes are also mobilized by transduction. Comparative genomic
46 analysis of the Se7942 and other cyanobacterial genomes identified xenologous genes
47 based on the combination of multiple approaches: best BLAST hit out of cyanobacteria,
48 absence of the ubiquitous octanucleotide HIP1 motif (Highly Iterated Palindrome 1) (8),
49 differences in codon usage, GC index and trinucleotide skews (9). Based on these
50 criteria, a majority of these genes (162 out of 253) probably originated from the
51 phylum proteobacteria. These data suggest that functional mechanisms of HGT must
52 exist, to provide a genetic bridge between phyla proteobacteria and cyanobacteria.
53 Conjugation has been used as a tool for introducing shuttle vectors from *E. coli* to both
54 pluricellular (several strains of heterocysts-forming *Anabaena* (10), non-heterocyst-
55 forming *Leptolyngbya* sp. strain BL0902 (11), akinetes, hormogonia and heterocyst-
56 forming *Fischerella muscicola* PCC 7414 and *Chlorogloeopsis fritschii* PCC 6912 (12))
57 and unicellular cyanobacteria (Se7942 (13, 14), several strains of marine *Synechococcus*
58 {Brahamsha, 1996}, *Prochlorococcus* strain MIT9313 (15) and *Synechocystis* sp. PCC
59 6803 (13)). These shuttle vectors were either based on the mobilization of the

promiscuous plasmid RSF1010 or in a ColE1-like origin of transfer (16). Although conjugative plasmids of several incompatibility groups were tested as helpers to mobilize these shuttle vectors, only IncP1-MOB_{P11} plasmids, such as RP4 and R751, were successful at transferring DNA from *E. coli* to cyanobacteria (17). Thus, up to now mobilizable shuttle vectors rely on IncP1 helper plasmids to be transferred to cyanobacterial recipients by conjugation (14, 16).

With few exceptions, proteobacterial conjugative plasmids can be grouped in five MOB families (MOB_P, MOB_F, MOB_Q, MOB_C and MOB_H) and three mating pair formation types (MPF_T, MPF_F and MPF_I) (18). Natural combinations MOB_{P11}-MPF_T (present in IncP1α plasmid RP4), MOB_{F11}-MPF_T (present in IncN plasmid pKM101 and IncW plasmid R388), MOB_{P12}-MPF_I (present in IncIα plasmid R64) and MOB_{F12}-MPF_F (present in IncFI plasmid F) were tested in this work to investigate the range of proteobacterial conjugative systems able to conjugate DNA to cyanobacteria. These plasmids were used as helpers to mobilize a series of BioBrick-compatible shuttle vectors containing a cognate MOB from *E. coli* to Se7942. Such vectors were helpful to define the functional replicon of plasmid pANL. Conjugation results showed that all tested MPF_T plasmids, regardless of their MOB type, were proficient to deliver DNA to cyanobacteria by conjugation, suggesting that plasmid conjugation from proteobacteria has contributed to the composition and evolution of Se7942 genome.

Materials and Methods

Strains and culture conditions. Strains used are detailed in Table 1. The original Se7942 strain we used was already cured of the endogenous plasmid pANS. Se7942 was cultured at 30ºC in BG11 medium (19) by bubbling 1% CO_2 with continuous light at 60µmol photons m^{-2} s^{-1}. *Leptolyngbya* PCC 7410, *Anabaena variabilis* ATCC 29413, *Plectonema boryanum, Nostoc punctiforme* PCC 73102, and *Nostoc punctiforme* ATCC 29133 were cultured in BG11 at 25ºC, 20 µmol photons m^{-2} s^{-1}, 10 rpm, and atmospheric CO_2 conditions. The *E. coli* strains used were BW27783, DH5α and β2150. They were grown at 37ºC under shaking in LB media. Strain β2150 was supplemented with 30µM diaminopimelic acid (DAP30). Antibiotics used for selecting cyanobacteria were neomycin 5 or 25µg/ml (Neo5 or Neo25), chloramphenicol 5 or 10µg/ml (Cm5 or Cm10), and streptomycin 10 or 50µg/ml (Sm10 or Sm50). Antibiotics used for selecting *E. coli* were: kanamycin 50µg/ml (Km50), rifampicin 50µg/ml (Rif50), chloramphenicol 25µg/ml (Cm25), nalidixic acid 20µg/ml (Nx20), and streptomycin 300µg/ml (Sm300).

Construction of vectors. Plasmids and oligonucleotides are listed in table 2 and supplementary table 1, respectively. The steps for construction of the shuttle vectors are depicted in Supplementary Figure S1, while dislodging vectors are described in Supplementary Figures S2 and S3. Details on the construction procedures are summarized in Supplementary Material and Methods.

Conjugation assays between *E. coli* and cyanobacteria and between *E. coli.* Biparental assays were used to conjugate DNA from *E. coli* to cyanobacteria. They were performed at 30ºC for Se7942 and at 25ºC for other cyanobacterial genera. For each conjugation, a Se7942 culture sample equivalent to 15µg chlorophyll (around $6x10^8$ cyanobacteria

cells/µg chlorophyll) were mixed with 100µl of serial dilutions of a 10^9 cells/mL *E. coli* culture. Conjugative mixtures were placed on top of a nitrocellulose filter, placed in turn onto a BG11 plate supplemented with 5% LB+DAP30 for 1 h in the dark. Then, the conjugative mixture was incubated for 24 h in the presence of light (60 µmol photons m^{-2} s^{-1}). Filters were later changed to fresh BG11 plates, incubated for an additional 24 h period and finally transferred to BG11+Neo25 under the same conditions. Transconjugant colonies became visible after 7-14 days incubation. Conjugation between *E. coli* cells was performed as previously described (20). Strains β2150 or BW27783 were used as donors, while DH5α was used as a recipient strain. Conjugation frequencies were expressed as the number of transconjugants per donor cell and were calculated as described by (21).

Natural transformation. Se7942 was transformed with plasmid pDEP30 following the protocol described by (22). Transformation mixtures were deposited onto nitrocellulose filters (Millipore) and incubated in BG11 plates at 30ºC with continuous light for 24 h. Transformants were selected in BG11+Cm10.

Dislodging assays and analysis of cyanobacterial transconjugant colonies. Dislodging vectors pDEP21 and pDEP23 were introduced in Se7942 by conjugation from *E. coli* using RP4 as helper plasmid. Individual transconjugant Se7942 colonies, carrying either pDEP21 or pDEP23, were grown in 250ml BG11+Neo5. Once cultures reached OD_{750}=2, 1.0 ml was transferred to 250ml BG11+Neo5. Serial dilutions were repeated for 42 or 64 generations of growth (for pDEP23 or pDEP21, respectively), when the axenic condition of these cultures was confirmed. Presence/absence of pANL was checked by PCR, using primers 31-32, 33-34 and/or 35-36, when appropriate.

Transconjugant colonies of Se7942 strain GRPS1 carrying pDEP23 were serially replicated in BG11+Neo5 plates to remove *E. coli* cells. Axenic cultures of GRPS1 carrying pDEP23 were transformed with pDEP30 plasmid DNA. The latter plasmid contains a *Synechocystis* sp. PCC 6803 gene *rps12* under the control of the *psbA1* gene promoter and a *cat* gene flanked by two 1 kb fragments located at both sides of the pANL maintenance region (Suppl. Material and Methods and Suppl. Figure S3). Thus, a double crossover between pANL and pDEP30 should remove pANL maintenance region. Transformant colonies were grown in BG11+Cm5 plates to improve segregation of the mutation, which was checked by PCR (30 cycles, 94ºC 60'', 50ºC 30'', 72ºC 30'') using primers 37-38 and 39-40 (Supplementary Figure S4). They were grown in liquid medium BG11+Neo5+Sm10 to favor displacement of the pANL derivative lacking the maintenance region (pDEP32) by pDEP23. Individual colonies recovered from BG11+Neo5+Sm50 plates were analyzed by PCR (30 cycles, 94ºC 60'', 50ºC 30'', 72ºC 30'') using primer pairs 33-34, and 35-36 to check for the absence of the pANL derivative.

To analyze the cyanobacterial transconjugants, single colonies were streaked-out twice in BG11+Neo5 plates to remove *E. coli* donors. Transconjugants were then grown in BG11+Neo5 up to OD_{750}=1-1.5. The axenic condition of these cultures was tested in LB+DAP30. Plasmid DNA was isolated using GenElute TM Plasmid Miniprep Kit (Sigma-Aldrich) and used to transform *E. coli* DH5α by electroporation. Km^R *E. coli* transformants were analyzed by electrophoresis of plasmid DNAs with *Eco*RI and *Pst*I in 1% agarose gel run in TBE 0.5x buffer (44.5mM Tris-borate/44.5mM Boric acid/1mM EDTA pH=8.2-8.4). Gels were stained with Real Safe (Real) and developed in a Gel Doc Imager (Bio-Rad).

Chromate resistance test. *E. coli* DH5α was independently transformed with cointegrate pDEP31 or vector pDEP6 by electroporation. Saturated cultures from single transformant colonies, grown in LB+Km50, were used to inoculate 96-well plates containing 150µl LB+Km50 per well and different concentrations of K_2CrO_4. Plates were incubated at 37ºC. Bacterial growth was followed by OD_{600} in a Victor3 plate reader (Perkin Elmer). Generation times were calculated as ln(2)/k, where k represents the growth rate and corresponds to the slope of the exponential growth phase (3 experiments, 8 replicas per experiment).

Results

Dislodging vectors to displace the indigenous Se7942 plasmid pANL. The 46.3 kb pANL plasmid, indigenous of Se7942, could potentially interfere with conjugation or stability of other plasmids, thus the interest of attempting pANL plasmid curing. Several shuttle plasmids were built, all based on a 1,395 bp DNA segment containing pANL putative origin of replication (23). The replication region (hereafter named *rep*_pANL) that was cloned to construct the pDEP vector series included an additional 18 bp fragment to complete the coding region of gene *anL57* (Figure 1A).

pDEP21 was built as a dislodging vector, containing the pANL replication region in order to remove plasmid pANL from Se7942 by vectorial plasmid incompatibility under selective pressure (24) (Figure 1B and Supplementary Figure S2). Since plasmid pANL encodes two toxin-antitoxin systems, both pANL antitoxin genes (*sepA1*, *sepA2)* were also included in pDEP21 to avoid killing pANL segregants. The dislodging plasmid pDEP21 also contains *oriT*_RP4 to allow conjugation from *E. coli* to cyanobacteria. Transconjugant colonies were subcultured in liquid BG11+Neo5 for 64 generations. Ten individual colonies were analyzed by PCR using primers that specifically hybridized to pANL and not to pDEP21. They all rendered amplicons congruent with the presence of pANL. Besides, plasmid DNA isolated from four independent colonies, transformed into *E. coli* and subjected to restriction analysis showed no differences in restriction pattern between the recovered plasmid DNAs and the original dislodging vector pEDP21 (Figure 1C), indicating that it could be autonomously maintained in Se7942 but was not able to completely displace pANL.

In another attempt to remove pANL, regions named *gap2* and *gap3*, described as essential by (23), were incorporated into the dislodging vector, thus producing pDEP23

(Supplementary Figure S2). This vector was introduced in Se7942 by conjugation. Transconjugant colonies were cultured for 42 generations in BG11+Neo5, plated out again and plasmid DNA was extracted from single colonies. Restriction analysis showed a cointegrate between plasmids pDEP23 and pANL. No instance of pANL curing was detected.

A third attempt to remove pANL was carried out by deleting its maintenance region, previously described to be essential for stable carriage of pANL in Se7942 (23). This region is composed of two toxin-antitoxin system cassettes (*sepA1-sepT1* and *sepA2-sepT2*), a set of partition genes (*parA* and *parB*) and an *orf* encoding a putative nucleotidyl-transferase (*anL30*). Deletion of this pANL segment (coordinates 20984 to 24487 in GenBank Accession No. AF441790) was carried out by homologous recombination in the Se7942 strain GRPS1, which is used for the construction of gene-replacement mutants (25). Plasmid pDEP30 (Supplementary Figure S3) was introduced into strain GRPS1 (pDEP23). The flanking areas of the pANL maintenance region surround genes *rps12* and *cat* in pDEP30, which confer a dominant Sm^S Cm^R phenotype. Deletion of the maintenance region was confirmed by PCR (Supplementary Figure S4) and the deleted pANL derivative was named pDEP32. To favor pDEP23 in the competition with pDEP32, GRPS1 was grown in Neo25+Sm50. No GRPS1 colonies free of pDEP32 were detected. All Sm^RNeo^R colonies tested contained a cointegrate between pDEP23 and pDEP32 (data not shown). In conclusion, all attempts at curing pANL failed, suggesting that this plasmid contains some genes that are essential for Se7942 viability under the tested conditions.

Mobilization of shuttle vectors from *E. coli* to Se7942 using different prototype proteobacterial conjugative plasmids. To test for the ability of different MPF systems

to transfer plasmid DNA by conjugation to cyanobacteria, we tested the mobilization of a series of plasmid *oriT*s by their cognate conjugative plasmids from *E. coli* to Se7942. Five prototype conjugative plasmids were tested (RP4, pKM101, R388, R64 and F) for transfer from *E. coli* to Se7942 (Figure 2). They represent five frequently found incompatibility groups (Inc) in □-proteobacteria, including four MOB subfamilies and three MPF types, thus comprising a representation of the diversity of proteobacterial mobility systems (18). First, mobilization of the shuttle vectors was tested by *E. coli* intraspecies crosses. As shown in Table 3 (column 3), all shuttle vectors were mobilized by their cognate conjugative plasmid between *E. coli* strains at frequencies of roughly 10^{-1} transconjugants per donor cell, indicating that the helper plasmids were efficient in promoting mobilization of their cognate mobilizable vectors. When mobilization of the same shuttle vectors was tested, but using Se7942 as a recipient (Table 3, column 4), those containing the *oriT*s of RP4, pKM101 and R388 produced cyanobacterial Neo^R transconjugants. RP4, pKM101 and R388 transconjugants were obtained at frequencies of about 10^{-3} in the mobilization of their cognate pDEP vectors. On the other hand, plasmids R64 and F showed conjugation frequencies undistinguishable from background levels. The temperature conditions used for Se7942 growth and mating were critical, since pDEP6 mobilization drastically dropped to background levels when mating temperature was shifted from 30ºC to 35, 37 or 40ºC (data not shown). To rule out natural transformation as the cause of Se7942 Neo^R colonies, a control assay was carried out by repeating the conjugation experiment using as donor an *E. coli* strain containing vector pDEP5. Since pDEP5 is devoid of *oriT*, transfer of its Neo^R marker can occur only by natural transformation. Results shown in Table 3 (column 5) indicate that natural transformation was extremely inefficient under these conditions, occurring at

frequencies not exceeding 10^{-8}. This in turn suggests that the number of transconjugants obtained using pDEP9 and pDEP10 as shuttle vectors (respectively mobilized by R64 and F) was within the range of natural transformation efficiency.

Finally, experiments to mobilize pDEP6 (the vector containing *oriT*_RP4) to other cyanobacterial genera using RP4 as a helper plasmid were carried out using a diversity of cyanobacterial recipients: *Leptolyngbya* PCC 7410, *Anabaena variabilis* ATCC 29413, *Plectonema boryanum, Nostoc punctiforme* PCC 7310 and *Nostoc punctiforme* ATCC 29133. No transconjugants were obtained in any of these cases (data not shown). Since RP4 could mobilize RSF1010 to different cyanobacteria {Koksharova, 2002}, it can be assumed that plasmid pANL (or its derivatives) cannot replicate in those cyanobacteria.

Analysis of pEDP6 in Se7942 transconjugants. To test if pDEP6 could be maintained as an autonomous replicon in Se7942, plasmid DNA was extracted from four transconjugant colonies, transformed to *E. coli* to amplify the amount of plasmid DNA, and analyzed with restriction enzymes. All plasmid preparations recovered from Se7942 rendered the same restriction pattern. It was different from the original pDEP6 plasmid and consistent with it being a cointegrate (named pDEP31), formed by homologous recombination between pDEP6 and pANL *rep* regions, as shown in Figure 3. Since pDEP31 contains the sulphate / chromate uptake operon of pANL (*srp* operon; (23, 26)), it should result in increased chromate sensitivity of *E. coli* cells containing cointegrate pDEP31. This was proven by analysis of chromate sensitivity (Supplementary Figure S5), which showed increased sensitivity to increasing amounts of chromate in DH5α (pEDP31) with respect to the control strain of DH5α (pDEP6). This result further indicates that the *srp* operon of Se7942 is adequately expressed in *E. coli*.

Discussion

The main purposes of this work were to determine which of the main conjugative systems from proteobacteria were able to transfer DNA to cyanobacteria by conjugation, to compare their relative efficiencies and to optimize the conjugation protocol. The finding that all MPF_T-type plasmids were efficient donors is probably the most relevant result. It indicates that conjugation of proteobacterial plasmids is a probable source of xenologous genes in Se7942, as suggested previously (9).

For a start, and in order to optimize Se7942 as a conjugation recipient, we attempted to remove the indigenous pANL plasmid from Se7942 by using a plasmid incompatibility strategy. This technique is based in the fact that, when two plasmids carrying the same origin of replication coexist in a cell, they become unstable due to interactions between their replication machineries. Vectorial incompatibility (one of the plasmids is lost with higher probability than the other (24)) was previously exploited to cure native plasmids from *Agrobacterium tumefaciens* (27, 28), *Bacillus anthracis* (29) or *Yersinia pestis* (30), among many other examples. To cure pANL, two dislodging vectors were built, pDEP21 and pDEP23. They contain the proposed minimal replication region of plasmid pANL, defined as discussed in the Supplementary Materials. When pDEP21 or pDEP23 were mobilized to Se7942 by RP4, pANL was not cured. Not even a pANL derivative lacking its maintenance region could be displaced by the dislodging vectors by applying selection to favor them in the competition. Thus, either the native pANL contains one or more essential genes that we did not include in our constructs, or the origin of replication described by (23) is incomplete and some additional functions provided by pANL are indispensable for replication or maintenance of the dislodging vector. DNA extraction of transconjugant colonies

showed that, in some cases, the pDEP derivative was still an autonomous replicon (Figure 1), while in others it formed a cointegrate with pANL (Figure 3). This result could occur if the incoming plasmid is unstable (by incompatibility), but cannot completely dislodge the resident plasmid, as discussed above. In our experiments, the restriction pattern of plasmid pDEP21 was not altered, indicating that it can remain as an autonomous plasmid in Se7942. On the other hand, plasmids pDEP6 and pDEP23, which contain the same *rep*_pANL as pDEP21, were always found forming cointegrates with pANL. Their different behavior remains unexplained. In any case, pANL could not be dislodged so all conjugation experiments were carried out in a Se7942 containing pANL.

Interphylum conjugation from *E. coli* to cyanobacteria is carried out in the laboratory solely by using an RP4-based helper plasmid (10, 16). Conjugation has been used as an alternative to transformation for the insertion of foreign genes in the Se7942 chromosome (22, 31). Mobilizable shuttle vectors were based either on plasmid pBR322 *oriT* (while MOB and MPF functions were provided *in trans* by a ColE1-like plasmid and RP4, respectively) (32), or on RSF1010, which is mobilized by RP4 (31). pBR322-based vectors were used to study if plasmids other than RP4 supported mobilization to *Anabaena* strains M-131 or PCC 7120 (17). Other IncP1 plasmids could mobilize such vectors, while IncW plasmids could not. It should be pointed out that the transfer efficiency of a given mobilizable plasmid depends on the conjugative plasmid used as helper, because the relaxosome provided by the mobilizable plasmid should make appropriate contacts with the coupling protein and the mating apparatus provided by the conjugative plasmid. For example, RP4 mobilizes ColE1 and RSF1010 between *E. coli* strains respectively 2 and 4 log more efficiently than the IncW plasmid

R388 (33). Thus, it is not surprising that IncW plasmids were unable to sustain conjugation to *Anabaena* in the referred conditions (17).

In this work we used plasmids RP4, pKM101, R388, R64 and F, which represent the diversity of conjugative systems in proteobacteria (18), as potential donors in conjugation from proteobacteria to cyanobacteria. The relevant shuttle vectors contained always the same *oriT* as the conjugative plasmid used as helper, to avoid the above mentioned inefficiencies due to heterologous interactions. This fact allows attention to be focused on the relative proficiency of each MPF type, not being distracted by the interactions between MPF and MOB modules. Actual transfer of a conjugative plasmid is an indication of two capabilities: first, its ability to invade the host population and second, its efficiency of replicating in that host. Even in the absence of replication, the invasive plasmid DNA can recombine with the host genome (given the appropriate recombination sites), thus integrating within the chromosome and becoming an integrative and conjugative element (ICE) (34). The host range and therefore the promiscuity of a plasmid can be inferred from sequence data (35). This type of analysis established that IncP1 plasmids (such as RP4) show a broad host range, in accordance to the fact that RP4 conjugates to cyanobacteria (10) and even to yeast (36). The same analysis indicated that IncW (R388) plasmids have a conjugative range perhaps broader than IncP, IncN (pKM101) plasmids have an intermediate range, and IncI (R64) or IncF (F) plasmids are narrow host range. Ample experimental evidence confirms these assumptions (37, 38).

Results shown in Table 3 indicate that all three conjugative plasmids with an MPF_T type transport channel (*i.e.*, RP4, pKM101 and R388) were able to achieve interphylum

conjugation from *E. coli* to Se7942. The other two plasmids, F and R64, which contain MPF_F or MPF_I type conjugation systems (Figure 2), could not introduce DNA in Se7942. These results suggest that different MPF types exhibit different abilities to conjugate to cyanobacteria. All transfer systems are able to transfer DNA to a much wider range of recipients than the replication ability of the vector plasmids (hence suicide vectors used for gene delivery by conjugation (39, 40), or the induction of SOS response towards invading DNA (41)). Thus, our results suggest that neither MPF_F nor MPF_I could deliver DNA to cyanobacteria while all three MPF_T plasmids tested, irrespective of their MOB type, could do it. Besides, the fact that pANL-based conjugation can occur through a variety of conjugative systems from *E. coli* to Se7942, but not to other cyanobacteria, suggests that pANL has a narrow replication host-range.

What is special about MPF_T? It is known that MPF_T, MPF_I and MPF_F share homology in some protein components, but also contain specific components that are signatures for each group (18). Thus, one possibility is that these differences could be responsible for the increased promiscuity of MPF_T type elements. Curiously, trans-kingdom transfer from bacteria to yeast and plants has only been reported for MPF_T conjugal types (42-44). There is scarce information of factors that determine the range of potential recipients of a given mating system. TraN, one of the MPF_F components of plasmid F, interacts with the outer membrane protein OmpA. This interaction results in stabilization of conjugation partners and is necessary for efficient mobilization (45). Adhesin PilV of the thin pilus encoded by plasmid R64 specifically interacts with the lipopolysaccharide of the recipient cells, determining recipient specificity (46, 47). Receptors similar to OmpA or specific lipopolysaccharide components might be lacking in cyanobacteria and thus prevent interphylum conjugation of MPF_F (F) or MPF_I (R64)

plasmids, being this a second alternative to explain MPF_T enhanced promiscuity. In any case, our results clearly demonstrate that conjugation to cyanobacteria is not limited to IncP1 plasmids but involves many MPF_T plasmids. These results broaden the number of conjugative systems that can be used for the genetic manipulation of cyanobacteria and explain the origin of Se7942 xenologous genes.

But genetic exchange between proteobacteria and cyanobacteria could occur in both directions. In fact, *E. coli* cells harboring a cointegrate between the shuttle vector pDEP6 and the endogenous Se7942 plasmid pANL, which contains the complete sulfur-regulated region of pANL, exhibited increased generation time in the presence of chromate (Supplementary Figure S5), as occurred in Se7942 (26). When encoded out of the context of the sulfur-regulated gene cluster, gene *srpC* conferred chromate resistance to *E. coli* (48). On the contrary, a Se7942 *srpC* deletion mutant exhibited lower doubling time than the wild type Se7942 in presence of chromate (26). The reproduction of a Se7942 phenotype in *E. coli* as a consequence of the presence of the pANL genome suggests that the genetic flow between proteobacteria and cyanobacteria could be bidirectional.

Finally, it should be emphasized that several parameters of the conjugation protocol were optimized during the course of this work to maximize conjugation frequencies to cyanobacteria. First, conjugation worked best at 30ºC, with few or no transconjugants obtained at higher temperatures (35 ºC, 37ºC and 40ºC were tested), although Se7942 growth rate is maximal at 41ºC. Second, *E. coli* strain β2150 was used as a donor. This strain is auxotrophic for DAP, which helped killing donor *E. coli* cells during the selection regime. Third, several donor:recipient ratios were assayed for each mating

experiment in order to obtain countable colonies in one of each series of filters. Other barriers to trans-phylum conjugation might exist that affect the efficiency of trans-phylum conjugation, including restriction/modification (RM) systems and CRISPRs among others. Se7942 contains both ((49, 50) and our unpublished data). In our conjugation assays, the incoming plasmid DNAs were not methylated for Se7942. It would be interesting to know if an appropriate modification, such as that devised for RP4-based conjugation to *Anabaena*, which was based on the methylases that protect DNA against restriction by *Ava*I, *Ava*II and *Ava*III (51), would result in still higher conjugation frequencies.

Acknowledgments

Work in FdlC laboratory was financed by the Spanish Ministry of Economy and Competitivity (MINECO) grant BFU2011-26608 and European Seventh Framework Program (282004/FP7-HEALTH-2011-2.3.1-2). MSM is a recipient of a PhD fellowship from MINECO. MPGB was a recipient of a JAE-Doc contract from CSIC, cofinanced by the European Science Foundation. The authors would like to thank Dr. Paula Tamagnini for allowing D.E. to conduct some conjugation experiments in her laboratory, the Pasteur Culture Collection (Institute Pasteur, Paris) for supplying Se7942 and Dr. Takahama for kindly sharing Se7942 strain GRPS1.

394 **Table 1. Bacterial strains used in this study**

Strains	Description/relevant characteristics	Reference
β2150	Δ*dapA*::(*erm-pir*) *thrB1004, pro, thi, strA, hsdS, lacZ* Δ*M15*, (F' *lacZ* Δ*M15 lacI*^{q}, *traD36 proA*+, *proB*+) [Em^{R} Sm^{R}]	(39)
BW27783	*lacI*^{q} *rrnB3* Δ*lacZ4787 hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568* DE(*araFGH*) ϕ(Δ*araEp* P_{CP8}-*araE*) [Nx^{R}]	(52)
DH5α	*F*$^{-}$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17*(r_K^- m_K^+), λ– [Nx^{R}]	(53)
Synechococcus elongatus PCC 7942	Wild-type strain lacking plasmid pANS (NC_007604 + NC_004073). Also known as *Anacystis nidulans* R2-SPc. Classified into the cyanobacterial section I.	(54) Pasteur Culture Collection
GRPS1	*S. elongatus* PCC 7942 with a mutation in *rps12-R43* [Sm^{R}]	(55)
Leptolyngbya sp. PCC 7410	Wild-type strain. Classified into the cyanobacterial section III.	Pasteur Culture Collection
Plectonema boryanum	Wild-type strain. Classified into the cyanobacterial section III.	Pasteur Culture Collection
Anabaena variabilis ATCC 29413	Wild-type strain (NC_007413 + NC_007410 + NC_007411 + NC_007412). Classified into the cyanobacterial section IV.	American Type Culture Collection
Nostoc punctiforme PCC 73102	Wild-type strain (NC_010628 + NC_010631 + NC_010632 + NC_010630 + NC_010633 + NC_010629). Classified into the cyanobacterial section IV.	Pasteur Culture Collection
Nostoc punctiforme ATCC 29133	Wild-type strain. Classified into the cyanobacterial section IV.	American Type Culture Collection

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398 **Table 2. Plasmids**
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Plasmid	Description	Reference
pRL443	Km^S RP4 derivative [Ap^RTc^R]	(51)
R388	[Su^RTp^R]	(56)
R64*drd11*	[Sm^RTc^R]	(57)
pKM101	[Ap^R]	(58)
pOX38	F derivative [Cm^R]	(59)
pSB1K3	Rep(pMB8) [Km^R]. Backbone for BioBrick parts cloning	http://parts.igem.org/Part:pSB1K3
pSB1C3	Rep(pMB8) [Cm^R]. Backbone for BioBrick parts cloning	http://parts.igem.org/Part:pSB1C3
pEXR91	Rep(pMB8)[Ap^RKm^R] containing gene *rps12* under the promoter of the *psbAI* gene	(25)
pDEP5	pSB1K3::(rep_{pANL}) [Km^R]	This study. Figure S1
pDEP6	pDEP11::(rep_{pANL}) [Km^R]	This study. Figures 2 and S1
pDEP7	pDEP12::(rep_{pANL}) [Km^R]	This study. Figures 2 and S1
pDEP8	pDEP13::(rep_{pANL}) [Km^R]	This study. Figures 2 and S1
pDEP9	pDEP14::(rep_{pANL}) [Km^R]	This study. Figures 2 and S1
pDEP10	pDEP15::(rep_{pANL}) [Km^R]	This study. Figures 2 and S1
pDEP11	pSB1K3::($oriT_{RP4}$) [Km^R]	This study. Figure S1
pDEP12	pSB1K3::($oriT_{pKM101}$) [Km^R]	This study. Figure S1
pDEP13	pSB1K3::($oriT_{R388}$) [Km^R]	This study. Figure S1
pDEP14	pSB1K3::($oriT_{R64}$) [Km^R]	This study. Figure S1
pDEP15	pSB1K3::($oriT_F$) [Km^R]	This study. Figure S1
pDEP16	pSB1K3::(*sepA1*) [Km^R]	This study. Figure S2
pDEP17	pSB1K3::(*sepA2*) [Km^R]	This study. Figure S2

pDEP18	pSB1K3::(Ptac) [Km^R]	This study. Figure S2
pDEP19	pDEP17::(*sepA1*) [Km^R]	This study. Figure S2
pDEP20	pDEP19::(Ptac) [Km^R]	This study. Figure S2
pDEP21	pDEP6::(Ptac-*sepA1-sepA2*) [Km^R]	This study. Figures 1 and S2
pDEP22	pSB1K3::(*gap2-3*)	This study. Figure S2
pDEP23	pDEP21::(*gap2-3*) [Km^R]	This study. Figure S2
pDEP24	pSB1K3::(HS1) [Km^R]	This study. Figure S3
pDEP25	pSB1K3::(HS2) [Km^R]	This study. Figure S3
pDEP26	pSB1K3::(*cat*) [$Km^R Cm^R$]	This study. Figure S3
pDEP27	pSB1K3::(*rsp12*) [Km^R]	This study. Figure S3
pDEP28	pDEP27::(*cat*) [$Km^R Cm^R$]	This study. Figure S3
pDEP29	pDEP25::(*cat-rsp12*) [$Km^R Cm^R$]	This study. Figure S3
pDEP30	pDEP29::(HS1) [$Km^R Cm^R$]	This study. Figure S3
pDEP31	pDEP6-pANL cointegrate [Km^R]	This study. Figure 3
pDEP32	pANL in which the maintenance region comprised between HS1 and HS2 has been replaced by *rps12-cat* [$Km^R Cm^R$]	This study. Figure S4

400

401

402 **Table 3. Conjugative frequencies between *E. coli* and from *E. coli* to Se7942**

Plasmids contained in the donor strain [a]	Inc MOB MPF type [b]	Mobilization frequency between *E. coli* [c]	Mobilization frequency from *E. coli* to Se7942 [c]	Mobilization frequencies of Δ*oriT* derivatives [d]
RP4+pDEP6 [e]	IncP1α MOB_{P11} MPF_T	$4.8x10^{-1}$ ($8.6x10^{-2}$–2.7)	$2.2x10^{-3}$ ($5.0x10^{-4}$–$1x10^{-2}$)	$5x10^{-9}$ ($9x10^{-10}$–$2x10^{-8}$)
pKM101+pDEP7	IncN MOB_{F11} MPF_T	$5.2x10^{-1}$ ($3.1x10^{-1}$–$8.4\ x10^{-1}$)	$1.2x10^{-3}$ ($4.5x10^{-4}$–$3.3x10^{-3}$)	$2x10^{-9}$ ($6x10^{-10}$–$1x10^{-8}$)
R388+pDEP8	IncW MOB_{F11} MPF_T	$5.0x10^{-2}$ ($1.9x10^{-2}$–$1.3x10^{-1}$)	$8.9x10^{-3}$ ($2.5x10^{-3}$–$3.1x10^{-2}$)	$1x10^{-8}$ ($6x10^{-9}$–$2x10^{-8}$)
R64*drd11*+pDEP9	IncI1α MOB_{P12} MPF_I	$8.5x10^{-2}$ ($6.1x10^{-2}$–$1.2x10^{-1}$)	$< 1x10^{-9}$	$4x10^{-10}$ ($8x10^{-11}$–$2x10^{-9}$)
F+pDEP10 [f]	IncFI MOB_{F12} MPF_F	$4.8x10^{-1}$ ($3.8x10^{-1}$–$5.9x10^{-1}$)	$< 1x10^{-9}$	$1x10^{-9}$ ($3x10^{-10}$–$7x10^{-9}$)

403

404 [a]: Donor strains were derivatives of *E. coli* strain β2150 containing the plasmids shown
405 in the first column. Strain BW27783 was used as donor in the case of plasmid R388,
406 since β2150 was inhibitory to R388 conjugation, due to an inhibitory effect of the
407 integrated F-plasmid ((39) and our unpublished results).

408 [b]: For a description of MOB and MPF types see (18).

409 [c]: Mobilization frequencies are the average of at least six experiments. They were
410 calculated by log conversion of frequencies (number of transconjugants per donor cell)
411 to obtain the mean and standard deviation values (in parentheses), which were
412 expressed as the anti-log of the calculated figures.

413 [d]: The same conjugation experiment from *E. coli* to Se7942 was conducted but using
414 the helper plasmids in combination with plasmid pDEP5, a non-mobilizable vector, to
415 determine the background level of Km^R Se7942 colonies that could arise in the mating

experiments due to natural transformation. Transformation frequencies were calculated as the number of transforming cells/number of donor cells.

[e]: pRL443, an RP4 derivative sensitive to kanamycin, was used as a helper plasmid.

[f]: pOX38, a Cm^R F derivative, was used as a helper plasmid.

421 ***Figure legends***

422 **Figure 1**. **pDEP21 autoreplicates in Se7942.** (A) The genetic organization of the
423 putative replication region of pANL is shown. The replication sequence according to
424 (23) is indicated by a white box outlined in black, while the segment used in this work
425 as *rep*_pANL is indicated by a grey box outlined in grey. (B) Genetic map of the
426 dislodging vector pDEP21. (C) Plasmid DNA recovered from Se7942 (pDEP21) cultured
427 in liquid BG11+Neo5 for 64 generations was restricted with *Eco*RI (E), and/or *Pst*I (P).
428 Plasmid DNA directly extracted from *E. coli* DH5α (pDEP21) without any passage
429 through Se7942 was used as a control in the restriction analysis.

430 **Figure 2. Shuttle vector series.** Mobilizable vectors are based on plasmid pSB1K3. All
431 contain the *rep* region of plasmid pANL. Each one includes the origin of transfer (*oriT*)
432 from a different prototype conjugative plasmid for which the Inc group, relaxase MOB
433 family, and MPF type are indicated.

434 **Figure 3**. **Analysis of transconjugant plasmid DNAs produced by pDEP6 conjugation to**
435 **Se7942.** The figure shows the electrophoretic analysis of DNA bands obtained by
436 restriction analysis of transconjugant plasmid DNA. (A) Plasmid DNA recovered from
437 Se7942 (pDEP6) transconjugants (i.e., pEDP31) was digested with *Eco*RI (E), and/or *Pst*I
438 (P) and developed by 1% agarose gel electrophoresis. Plasmid pDEP6 DNA (isolated
439 from *E. coli* DH5α) was used as a control in the restriction analysis. (B) *In silico*
440 restriction analysis of pDEP6-pANL cointegrate pDEP31 assuming a single crossover
441 across the pANL *rep* region. (C) Schematic representation of the single crossover
442 leading to pDEP31 formation, according to the results obtained in panels A and B. The

443 position of the single *Pst*I site present in pANL replication region, which is not present

444 in pDEP6, helps to decide the direction of pDEP6 insertion.

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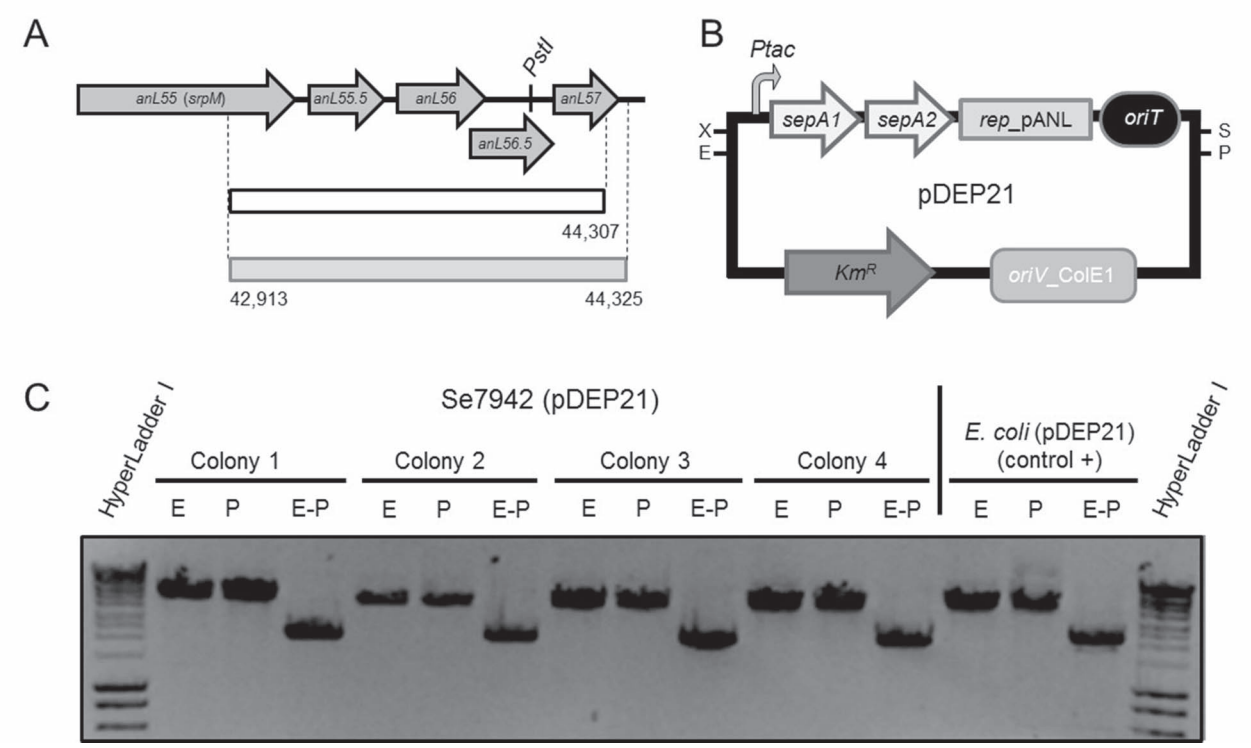

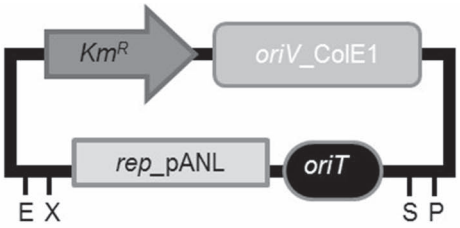

Shuttle vector name		Inc group	MOB subfamily	MPF type
pDEP6	RP4	IncP1α	MOB_{P11}	MPF_T
pDEP7	pKM101	IncN	MOB_{F11}	MPF_T
pDEP8	R388	IncW	MOB_{F11}	MPF_T
pDEP9	R64	IncI1α	MOB_{P12}	MPF_I
pDEP10	F	IncFI	MOB_{F12}	MPF_F