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A Diaminopimelic Acid Auxotrophic Escherichia coli Donor Provides Improved Counterselection Following Intergeneric Conjugation with Actinomycetes

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2 **Counterselection Following Intergeneric Conjugation with Actinomycetes**

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17 **Abstract**

18 Considering the medical, biotechnological, and economical importance of actinobacteria,
19 there is a continuous need to improve the tools for genetic engineering of a broad range
20 of these microorganisms. Intergeneric conjugation has proven to be a valuable yet
21 imperfect tool for this purpose. The natural resistance of many actinomycetes against
22 nalidixic acid (Nal) is generally exploited to eliminate the sensitive *Escherichia coli*
23 donor strain following conjugation. Nevertheless, Nal can delay growth and have other

unexpected effects on the recipient strain. To provide an improved alternative to antibiotics, we propose a post-conjugational counterselection using a diaminopimelic acid (DAP) auxotrophic donor strain. The DAP-negative phenotype was obtained by introducing a *dapA*-deletion into the popular methylase-negative donor strain ET12567/pUZ8002. The viability of ET12567 and its $\Delta dapA$ mutant exposed to DAP deprivation or Nal selection were compared in liquid pure culture and after mating with *Streptomyces coelicolor*. Results showed that death of the *E. coli* $\Delta dapA$ Nal-sensitive donor strain occurred more efficiently when subjected to DAP deprivation than when exposed to Nal. Our study shows that post-conjugational counterselection based on DAP deprivation circumvents the use of antibiotic and will facilitate the transfer of plasmids into actinomycetes with high biotechnological potential, yet currently not accessible to conjugative techniques.

Key words

intergeneric conjugation, counterselection, diaminopimelic acid, actinomycetes.

Résumé

Étant donné l'importance médicale, biotechnologique et économique des actinobactéries, il y a un besoin constant d'améliorer les outils permettant de modifier génétiquement un large éventail de ces microorganismes. La conjugaison intergénérique est un outil éprouvé pourtant imparfait à cet effet. La résistance naturelle de plusieurs actinomycètes à l'acide nalidixique (Nal) est généralement exploitée pour éliminer la souche donneuse d'*Escherichia coli* sensible, à la suite d'une conjugaison. Toutefois, le Nal peut affecter la

croissance et avoir des effets inattendus sur la souche réceptrice. Pour offrir une alternative à l'antibiotique Nal, nous proposons une technique de contre-sélection en utilisant une souche donneuse auxotrophe pour l'acide diaminopimélique (DAP). Le phénotype DAP-négatif a été obtenu en introduisant la délétion de *dapA* chez la populaire souche donneuse méthylase-négative ET12567/pUZ8002. La viabilité d'ET12567 et de son mutant $\Delta dapA$, exposé soit à la sélection par carence en DAP ou par sensibilité au Nal, ont été comparées en culture pure liquide et après une conjugaison avec *Streptomyces coelicolor*. Les résultats démontrent que la mortalité chez *E. coli* $\Delta dapA$ sensible au Nal se produit plus efficacement en absence de DAP qu'en présence de Nal. Notre étude démontre que cette méthode de contre-sélection post-conjugationnelle permet d'éviter l'utilisation d'antibiotique et facilitera le transfert de plasmides chez des actinomycètes ayant un potentiel biotechnologique, pour lesquels les techniques de conjugaison ne sont pas encore accessibles.

Mots clés

conjugaison intergénérique, contre-sélection, acide diaminopimélique, actinomycètes.

Introduction

The actinomycetes comprise a diverse group of Gram-positive, filamentous bacteria renowned for being the most important producers of antibiotics and other secondary metabolites such as immunosuppressants, antitumor compounds, antivirals, and antiparasitic agents (Bibb 2013). These commercially valuable compounds are largely used in agriculture, food production and pharmacology. Consequently, there is a constant need to develop new approaches for the genetic manipulation of a wide range of

industrially relevant actinomycetes. Genetic tools are crucial for the identification and functional analysis of biosynthetic pathways involved in metabolite production, the optimization of the expression of genes of interest, as well as the engineering of overproducing strains (Baltz 2011).

Bacterial conjugation has proven to be a valuable tool, given the large amount of genetic material that can be transferred per conjugation event and the broad host range of some conjugative plasmids. Intergeneric conjugation between *Escherichia coli* and a variety of Gram-positive bacteria was initially demonstrated by (Trieu-Cuot et al. 1987). Afterwards, (Mazodier et al. 1989) developed the first protocol for conjugation between *E. coli* and *Streptomyces*. Ever since, conjugative transfer of plasmids has often been used efficiently to introduce foreign DNA into multiple actinobacterial genera such as *Corynebacterium* (Schäfer et al. 1990), *Saccharopolyspora* (Matsushima et al. 1994), *Actinomaduras*, *Arthrobacter*, *Micromonospora*, *Nocardia*, *Rhodococcus* (Voeykova et al. 1998), *Amycolatopsis* (Stegmann et al. 2001), *Nonomuraea* (Stinchi et al. 2003), and *Actinoplanes* (Heinzelmann et al. 2003). Unfortunately, despite the progress made in these conjugation-based methodologies, several inherent limitations remain, including the ability to counterselect the exconjugants against the donor strain. The natural resistance of many actinobacteria to nalidixic acid (Nal) is generally exploited to eliminate the sensitive *E. coli* donor strain following conjugation. Nevertheless, to circumvent the use of antibiotics, which could have unexpected effects on the recipient strain, we exploited an alternative counterselection approach.

93 In *E. coli*, the first enzyme of the diaminopimelate pathway is dihydrodipicolinate
94 synthetase (DHDPS) encoded by the *dapA* gene (Richaud et al. 1986). This enzyme is
95 required for the biosynthesis of D,L-diaminopimelic acid (*meso*-DAP), which provides
96 the cross-linkage of peptidoglycan in many Gram-negative and mycobacterial cell walls.
97 Furthermore, DAP serves as a direct precursor of lysine, one of the 20 essential amino
98 acids. Previous work showed that disruption of this metabolic pathway leads to bacterial
99 cell death and renders *E. coli* unable to grow in media lacking DAP (Born and Blanchard
100 1999). The successful use of auxotrophic donors to facilitate the selection of
101 exconjugants has been exploited in conjugations with *Bartonella* (Dehio and Meyer 1997,
102 Dehio et al. 1998), *Vibrio* (Demarre et al. 2005), *Burkholderia* (López et al. 2009), and
103 *Pseudomonas* (Thoma and Schobert 2009). However, these auxotroph strains are DNA
104 methylation proficient, which is inappropriate for conjugation with actinomycete methyl-
105 restricting hosts, since even hemi-methylated DNA represents a barrier to the
106 introduction of heterologous DNA (Flett et al. 1997). Therefore, we constructed a $\Delta dapA$
107 mutant of the methylation-deficient *E. coli* ET12567 to achieved counterselection easily,
108 without affecting the recipient strains by the addition of antibiotic following conjugation.

109 **Materials and methods**

110 **Bacterial strains and culture conditions**

111 Methylation-deficient *E. coli* ET12567 (*dam-13::Tn9*, *dcm-6*, *hsdM*, *hsdS*) was used as
112 recipient for transduction of the $\Delta dapA$ mutation and as the donor strain in intergeneric
113 mating experiments. ET12567 harbors the non-transmissible helper plasmid pUZ8002, a
114 derivative of RK2 able to mobilize other plasmids efficiently (MacNeil et al. 1992).
115 Routinely, *E. coli* ET12567 was grown at 37 °C in LB medium, with shaking (225 rpm)

or on LB agar. *Streptomyces coelicolor* A3(2) M145 (SPC1⁻, SPC2⁻) was cultured at 30 °C on SLM3 agar (DeWitt 1985) for the preparation of spore stocks, which were subsequently stored in 20% glycerol at -20 °C. Mannitol soya flour (MS) medium (Kieser et al. 2000) was used for the mating assays and subsequent selection of exconjugants and total count of recipients. Exconjugants chosen for further analysis were spread onto tryptic soy agar (TSA) containing apramycin (selectable marker carried by the mobilizable plasmid pSET152) and then, grown at 30 °C with shaking (225 rpm) in tryptic soy broth (TSB). To allow growth, culture media for *E. coli* ET12567 Δ dapA were supplemented with 0.3 mM DL- α,ϵ -diaminopimelic acid (DAP). DAP was also added to growth medium in some control experiments with the wild-type ET12567 strain. When required, the antibiotics were used at the following concentrations: 50 μ g/ml apramycin sulfate (Apr), 25 μ g/ml chloramphenicol (Cam), 200 μ g/ml erythromycin (Erm), 25-50 μ g/ml kanamycin sulfate (Kan), 25 μ g/ml nalidixic acid sodium salt (Nal).

Transduction

The deletion of the *dapA* gene in *E. coli* ET12567/pUZ8002 was introduced by P1vir transduction using DPL12 (*E. coli* MG1655 Δ dapA::(*erm-pir*)) (Carraro et al. 2014) as donor. The latter strain bears a 185-bp deletion within the reading frame of the *dapA* gene (Richaud et al. 1993) further disrupted by a synthetic *erm-pir* operon (Demarre et al. 2005). Transductants were recovered after 24 h of growth on LB agar containing Kan (25 μ g/ml), Cam and Erm to select for pUZ8002, the *dam* mutation and the Δ dapA mutation,

respectively. DAP was also added to the medium to allow growth of the $\Delta dapA$ strains. DAP-deficient mutants were tested for the lethal phenotype in the absence of exogenously added DAP. The deletion of *dapA* in two mutants was further confirmed by PCR analysis using primers flanking the deletion region (Fig. S1). The amplification and sequencing of a 1820-bp fragment of the *rpoD* gene, encoding the RNA polymerase primary σ^{70} factor was used as a PCR positive control (Fig. S1).

Growth curves

The growth characteristics of *E. coli* strains were compared in LB medium supplemented or not with various combinations of DAP or Nal. The strains were initially grown in 5 ml LB medium (supplemented with DAP for the $\Delta dapA$ mutant) for 18 h, then washed twice in LB medium and brought to equal cell densities (OD_{600nm} of 0.1) in 50 ml of fresh LB medium with appropriate supplements. Growth was monitored for 22h35 (1355 min) at 37 °C with continuous shaking (225 rpm). Each condition was tested in three biological replicates and the growth curves were based on duplicate measures of OD_{600nm} for each sample. The ability to grow into colony forming units (CFUs) was estimated by serial dilutions of several time points of the growth curve culture, and plating on LB agar (supplemented with DAP for the $\Delta dapA$ mutant). The count of CFUs was used to determine the mean % of survival ($\text{Survival (\%)} = \text{number of CFUs at time X} / \text{number of CFUs at time 0} \times 100$).

Fluorescence microscopy

To estimate the cell wall integrity, *E. coli* cells were collected at three time points during growth (0, 210, and 390 min) and stained for 15 min in the dark at room temperature with LIVE/DEAD[®] BacLight Bacterial Viability Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The two nucleic acid stains were used as a 1:1 mixture of the green fluorescent SYTO 9 and the red fluorescent propidium iodide (PI). When appropriate, samples were diluted 1:10 or 1:100 in LB medium prior to the staining procedure to allow cell counting by fluorescence microscopy. A volume of 20 µl of the stained bacterial suspension was trapped between a slide and a 20 × 30 mm cover glass. Pictures were taken after 20 min to allow the deposition of the cells. Images were acquired on an Axio Imager M1 (Zeiss) using a 40X objective and appropriate excitation and emission filters for each fluorescent dye. Experimental error was reduced with the acquisition of eight images (random fields) per slide per dye (n=3). Image pre-processing and segmentation were performed with CellProfiler software version 2.1 (Carpenter et al. 2006). Threshold was applied to identify PI positive cells and the percentage of positive cells per image was determined. For each condition, the mean and standard error of the mean (SEM) were calculated.

Intergeneric conjugation

E. coli ET12567 (pUZ8002/pSET152) or its $\Delta dapA$ mutant were used as donors in intergeneric conjugations. The model actinomycete *Streptomyces coelicolor* A3(2) M145 was selected as recipient since it is a well characterized strain often used in standard experiments (Blaesing et al. 2005). The established protocol for conjugation (Kieser et al. 2000) was adapted with the following modifications. The pSET152 vector (Apr^R)

(Bierman et al. 1992) was used to direct its site-specific integration into the bacteriophage ϕ C31 attachment site in the chromosome of *S. coelicolor*. Approximately 10^8 *Streptomyces* spores were suspended in 500 μ l of 2 $\times$ YT broth, heat shocked at 50 °C for 10 min and cooled to room temperature. The spore suspensions were spread onto a polycarbonate membrane filter (PCTE, 0.2 μ M, 25 mm, Main manufacturing, MI, USA), placed onto MS agar with 10 mM $MgCl_2$, supplemented or not with 0.3 mM DAP. The plates were incubated 30 min at 30 °C to allow spore germination. Then a drop of overnight cultures containing $\sim 5 \times 10^7$ cells of donor strain was added to the germinated spores and mating was performed at 30 °C for 5 or 20 h. Total donor CFUs were quantified at the beginning of the mating by plating appropriate serial dilutions on LB agar $\pm$ DAP. Mating partners were recovered in 1 ml of 2 $\times$ YT broth and 100 μ l of serial dilutions were plated onto selective media to quantify the recipient and exconjugant CFUs. Counterselection of ET12567 strain was carried out by using 25 μ g/ml Nal, while counterselection of the $\Delta dapA$ mutant was achieved by adding either (i) 25 μ g/ml Nal with 0.3 mM DAP or (ii) without Nal nor DAP. The plates were incubated for 18-20 h and then overlaid with 2.5 ml soft nutrient agar with or without Apr for exconjugants selection and total count of recipient CFUs, respectively. Incubation was continued 3 to 5 days until CFUs of exconjugants appeared. To estimate the frequency of spontaneous Apr-resistant mutants, control conjugation mixes were processed as above but with the addition of *E. coli* donor cells not carrying pSET152. The frequency of exconjugant formation was obtained by dividing the number of exconjugant CFUs by the number of donor CFUs. Exconjugants were further cultured on selective TSA and analyzed by PCR for site-specific integration of pSET152 into the ϕ C31 *attB* site.

Comparison of DAP and Nal counterselection

In order to compare the efficiency of both counterselection approaches, the viability of ET12567 and its $\Delta dapA$ mutant donor strains was estimated over time following a 5 h mating procedure. To do this, mating partners were resuspended from the membrane filter in 1 ml 2×YT broth and 100 µl samples were plated in triplicate onto MS medium supplemented or not with DAP, and DAP + Nal for the $\Delta dapA$ mutant or onto MS medium with or without Nal for ET12567. The plates were incubated at 30 °C and the viability of *E. coli* was monitored at 0, 2, 4, and 6 h after conjugation by harvesting the cells from the plates. For this purpose, plates were washed twice with 5 ml LB medium and gently scraped with a polypropylene cell spreader to retrieve cells. After centrifugation at 500 g for 10 minutes and removal of the supernatant, the cells were resuspended in 1 ml of LB medium. Serial dilutions were plated on LB agar supplemented with Kan (antibiotic marker carried by pUZ8002) and with or without DAP to allow the growth of the donor strain only. CFUs were counted after 18 h of incubation at 37 °C. Four replicates were performed for each viability count assay.

DNA isolation and molecular techniques

Preparation and transformation of competent *E. coli* cells were performed as described by Sambrook and Russell (2001). *Streptomyces* genomic DNA was isolated using the salting out procedure (Kieser et al. 2000), while *E. coli* genomic DNA was isolated as described by Rasmussen et al. (2008). PCRs were used to detect the presence of the *rpoD* and *dapA* genes in *E. coli* as well as the *acc(3)IV* gene segment (Apr^R) and the fragments

confirming the site-specific integration of pSET152 in *S. coelicolor* exconjugants (*attR*, *attL*). PCRs were carried out in total reaction volumes of 50 µl with Easy-A High Fidelity *Taq* polymerase (Stratagene-Agilent Tech., Mississauga, ON, Canada) according to the manufacturer's instructions. The PCR cycles comprized pre-incubation of 2 min at 95 °C followed by 30 cycles of 40 sec at 95 °C, 30 sec at 50-65 °C (depending on primers used), and 1 min at 72 °C with a final extension step of 7 min at 72 °C. All oligonucleotides (Table 1) were provided by Integrated DNA Technologies (Coralville, IA, USA). When needed, PCR products were purified using the QIAquick gel extraction kit (QIAGEN, Mississauga, ON, Canada) or cloned into pCR2.1TOPO (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Sequencing of PCR products was performed at Genome Quebec Innovation Center of McGill University (Montreal, QC, Canada). The DNA sequences were compared with the GenBank DNA sequence database using the BLASTN program (Altschul et al. 1990).

Results

Viability of *E. coli* ET12567 and its $\Delta dapA$ mutant in pure liquid culture

In order to compare the efficiency of the DAP and Nal selections, three approaches were assessed with pure cultures of *E. coli* ET12567 and its $\Delta dapA$ mutant: cell density in LB medium, CFU counts on LB agar, and verification of the membrane integrity using the SYTO 9 and PI dyes. The growth characteristics of *E. coli* ET12567 and its $\Delta dapA$ mutant were compared at several time points after either addition of Nal (+ DAP) or the DAP deprivation treatments. The growth of *E. coli* ET12567 does not seem to be influenced by the presence or absence of exogenously supplied DAP, since the growth

rates remained the same in both conditions (Fig. 1A) even when the cells were treated with Nal (data not shown). As expected, the growth of the $\Delta dapA$ mutant was completely abolished in LB devoid of DAP, while its growth rate was almost restored to wild-type level when the medium was supplemented with DAP. In the case of selection by Nal sensitivity, the cell densities of ET12567 and its $\Delta dapA$ mutant both remained very low and relatively stable over time (Fig. 1A), but the viable count decreased after 75 min (Fig. 1B). Nevertheless, when cultures were further incubated for 22h35, cell density slightly increased in the media containing Nal (OD_{600nm} 0.37 ± 0.007 for $\Delta dapA$ and 0.94 ± 0.19 for ET12567), indicating that some cells were still alive and were beginning to proliferate. It was not the case in the medium lacking DAP, where the $\Delta dapA$ mutant viability sharply declined over time (about a tenfold decrease between each time point in Fig. 1B), suggesting that bacteria underwent cell lysis in the absence of DAP. The results demonstrate that the decrease of cell viability caused by Nal occurred much later and less efficiently than in conditions of DAP deprivation. Those results are also supported by the proportion of survival (Table 2), where the $\Delta dapA$ mutant showed a viability of $6.2 \pm 1.5\%$ in LB medium supplemented with DAP + Nal, and only $0.1 \pm 0.007\%$ in DAP-free medium after 210 min of culture. In the conditions used in our assays, taking advantage of DAP auxotrophy instead of Nal sensitivity is more efficient at stopping cell proliferation, supporting that the use of auxotrophic donors is a more effective counterselection approach.

To support the effects of DAP deprivation and Nal sensitivity on cell density and CFUs counts, the membrane integrity of *E. coli* ET12567 and its $\Delta dapA$ mutant was assessed

276 using the LIVE/DEAD[®] BacLight Bacterial Viability Kit. Although the staining method
277 employed cannot prove viability (Netuschil et al. 2014), stained cells are referred to as
278 live or dead cells and the relative proportions are reported in Fig. 1C. Live/Dead ratios of
279 cells harvested at 0, 210, and 390 min (Fig. S2) were achieved on the basis of red-
280 fluorescent PI staining, since this dye penetrates only cells with damaged membrane as
281 opposed to SYTO 9, a green-fluorescent dye that stains all the cells. In LB medium
282 without selection agents, the proportion of live cells in the population of ET12567 strain
283 was stable across the three time points tested ($89.6 \pm 6.2\%$ of green-only labeled cells).
284 The percentage of live $\Delta dapA$ mutant treated with Nal in the presence of DAP decreased
285 to 65% after 210 min. A similar decrease was observed as early as time point 0 min for
286 the $\Delta dapA$ mutant cultivated in DAP-less medium. At this time point, the cells were
287 observed after about 40 minutes from sampling – the period of time necessary for
288 staining and microphotography. This suggested that deprivation of DAP led to cell death
289 within a very short period of time. Once more, the results demonstrate that the mortality
290 rate occurred faster when cells were deprived of DAP than when treated with Nal.
291 Although these observations are consistent with our previous results, it is not known
292 whether the staining of either of the dyes could be distorted, irrespectively of the live or
293 dead status of the cell, given that the $\Delta dapA$ mutation could have altered the cell wall.

294

295 **Evaluation of *E. coli* ET12567 and its $\Delta dapA$ mutant post-conjugative viability**

296 In order to quantify viability of ET12567 and its $\Delta dapA$ mutant following a mating
297 procedure of 5 h, a CFUs counting approach was used. The % of survival of *E. coli* were
298 quantified after 0, 2, 4, and 6 h post-conjugation on MS medium with or without DAP, or

in the presence of Nal (+ DAP for the $\Delta dapA$ mutant). As expected, the addition of DAP allowed exponential growth of the $\Delta dapA$ mutant over time, which was comparable to the ET12567 strain in absence of selection (Fig. 2). When Nal was also added to the medium, the survival of the $\Delta dapA$ mutant was substantially reduced after the first 2 h post-conjugation ($25.7 \pm 2.6\%$), but the mortality rate slowed down during further incubation (Fig. 2). Results obtained with the ET12567 strain were comparable to those of the $\Delta dapA$ mutant, demonstrating that the latter strain does not respond differently to Nal treatment compared to the wild type strain. On the other hand, DAP deprivation following conjugation greatly reduced the survival of the $\Delta dapA$ mutant after 2 h of incubation ($13.5 \pm 6.1\%$), while there were less than $1.3 \pm 0.4\%$ of alive cells after 6 h. Consequently, death due to lack of DAP occurred significantly faster than that caused by nalidixic acid sensitivity.

DAP counterselection is suitable for intergeneric conjugation

Mating experiments were carried out using either Nal sensitivity or DAP auxotrophy counterselection against *E. coli* $\Delta dapA$ mutant. Following a 5-h mating procedure, the frequency of exconjugant formation was about 3.5 times higher for cells plated on MS containing DAP and Nal compared to DAP-less plates. Extension of the mating contact, to up to 20 h, resulted in increased frequencies of exconjugant formation for both selection approach. Also, when the conjugation period was long-lasting, the difference between the two counterselection techniques became no longer significant (Fig. 3A). Nonetheless, since some streptomycete mycelia started to sporulate on the filter, the exconjugant frequency cannot be used to compare the differences between the two

counterselection techniques. Exconjugant frequencies obtained with the ET12567 strain were similar to those of the $\Delta dapA$ mutant when sensitivity to Nal was used for counterselection. In all the conditions used, no Apr^R CFUs were observed in control matings between *S. coelicolor* and *E. coli* ET12567/pUZ8002 ($\Delta dapA$) without the mobilizable plasmid pSET152.

Exconjugants were further cultivated on selective TSA plates to confirm the conjugative transfer and stable maintenance of pSET152. Transfer and subsequent integration of pSET152 into the chromosome of *S. coelicolor* was confirmed in all five exconjugants examined by PCR amplification of the Apr resistance gene fragment and the right and left attachment sites (*attR*, *attL*) of ϕ C31 (Fig. 3B). While recovery of a few *E. coli* CFUs was sporadically observed on plates with Nal selection, it was not the case when using DAP deprivation. To evaluate the possible persistence of *E. coli* cells when cultivating recipients strains, exconjugants were transferred in selective TSB with simultaneous Nal selection or in the presence of DAP. After retrieving total genomic DNA, PCR amplification of the *E. coli rpoD* gene was performed, but *rpoD* fragment could not be detected in any of the conditions (data not shown).

Discussion

Bacterial conjugation is one of the most popular methods used to introduce DNA into actinomycetes (Flett et al. 1997). Here, we propose a post-conjugation counterselection based on DAP deprivation rather than antibiotic selection. This study is the first systematic comparison of two different strategies to eliminate the *E. coli* donor following

an intergeneric mating procedure with actinobacteria. While antibiotics, such as phosphomycin (Blaesing et al. 2005), and gentamycin (Li et al. 2003), have been used for counterselection following intergeneric conjugation, nalidixic acid is the most commonly used with actinomycete recipient strains. This quinolone antibiotic inhibits DNA gyrase, an enzyme important for maintaining the topology of genome and essential for chromosomes condensation and partitioning at cell division (Champoux 2001), resulting in DNA synthesis inhibition and bacterial death. Our results revealed that Nal is less efficient than DAP deprivation for selecting against *E. coli* cells in the conditions of our mating experiment. Previous studies showed that selectable phenotypic variations exist in isogenic populations, without significant genotypic variation, allowing a fraction of the cells to survive antibiotic selection, while most died (Adam et al. 2008, Sánchez-Romero and Casadesús 2014). Moreover, the mutation rate for a single gene occur at approximately 2×10^{-7} or 3×10^{-9} per division in different *E. coli* K-12 strains (Drake 1991). Even if the rate at which antibiotic resistance can arise is much lower, there are preexisting spontaneous Nal-resistant mutants in a population. Both these phenomena could explain the increased optical density of the cultures with Nal selection after 22h35 of growth (Fig. 1A), as well as the appearance of *E. coli* CFUs on selective MS medium following counterselection with Nal after a mating procedure. Therefore, our results suggest that phenotypic heterogeneity of bacterial population limits the effectiveness of Nal counterselection against *E. coli* donors.

An easy way to circumvent the use of nalidixic acid is to use an auxotrophic donor strain. Many type of auxotrophies exist but most of them are alleviated by compounds found in

rich media (Demarre et al. 2005). Only a few auxotrophies can be counterselected on rich complex media, such as mutants requiring 5-aminolevulinic acid (Thoma and Schobert 2009) or diaminopimelic acid (Demarre et al. 2005) for growth. DAP auxotrophs are well-characterized and have been used for different purposes for many years. In *E. coli*, genes involved in the DAP metabolic pathway have been inactivated, such as *dapA*, *B*, *C*, *D*, *E* (Bukhari and Taylor 1971, Dehio et al. 1997, Dehio et al. 1998). Other genes have been targeted in other strains: *asd* in *Salmonella* (Curtiss et al. 1989, Galán et al. 1990), *Neisseria* (Hatten et al. 1993), and *Shigella* (Sizemore et al. 1995), *ask* in *Mycobacterium* (Pavelka et al. 1996), *dapB* in *Pseudomonas* (Rediers et al. 2003), *asd* and *dapB* in *Burkholderia* (Norris et al. 2009), *dapA* in *Yersinia* (Bland et al. 2011). All such mutants are unable to grow on media without DAP supplementation. The use of a $\Delta dapA$ mutant is suitable for counterselection, given that the bacterial growth is inhibited by two mechanisms. First, *meso*-DAP is a major constituent of peptidoglycan, acting as a cross-linking agent in the bacterial cell wall. Its absence directly compromises cellular integrity. Second, DAP serves as a direct precursor of lysine thus, leading to the inability of a $\Delta dapA$ mutant to synthesize proteins (Pavelka and Jacobs 1996). It is possible that a fraction of the bacterial population could act as 'scavengers' and uptake the peptidoglycan components released from neighbouring cells which underwent lysis. However, it does not seem to be the case, probably because of the cumulative effects of DAP deprivation on protein synthesis inhibition. Thereby, the absence of DHDPS enzyme confers an absolute dependence on exogenous DAP for viability and growth. Unlike *Nal*, for which some natural resistance could be seen, no spontaneous reversion of the DAP-dependent growth is possible. Therefore, DAP auxotrophy is a more convenient and effective

counterselection approach given that it can be easily done on commonly used media, as they are based on yeast or animal extracts which lack DAP (Demarre et al. 2005). Also, the selection of exconjugants can be obtained under optimal growth conditions in significantly shorter time (Thoma and Schobert 2009), which is especially interesting for slow-growing bacteria.

When comparing both counterselection approaches following a 5-h mating procedure, it seems that using Nal selection is favorable since the exconjugant frequency was about 3.5 times higher than in the condition of DAP deprivation. Those results were unusual since previous investigations have demonstrated that the addition of Nal to mating pairs of sensitive donor and resistant recipient in liquid resulted in an immediate arrest of conjugative plasmid transfer in *E. coli* (Barbour 1967, Bouck and Adelberg 1970, Hane 1971). However, in order to perform selection after intergeneric conjugation, cells were plated on solid medium rather than being in a liquid mixture. Thus the entry of Nal in the cells could be less efficient or delayed, allowing some donor cells to reinitiate conjugative transfer of DNA. It is also possible that the actual concentration of Nal on plates was lower than expected due to the soybean component in MS agar known to absorb some antibiotics (Thamchaipenet et al. 2013). Therefore, Nal is less efficient than DAP deprivation to kill *E. coli* cells in the context of a mating experiment. Still, for actinobacteria which generally present low exconjugant frequencies, a long-lasting mating procedure can be appropriate to benefit from the DAP counterselection technique. To further modify our current donor strain, one could consider introducing a *recA* mutation to avoid fortuitous recombination between the helper plasmid pUZ8002 and the

plasmid that is meant to be mobilized, and/or integrating pUZ8002 into the donor genome.

In this work, we demonstrate the usefulness of a $\Delta dapA$ mutant as an alternative to antibiotic counterselection in bacterial intergeneric conjugation. This counterselection approach makes conjugation accessible to a wider range of bacterial species potentially sensitive to Nal, like *Micromonospora* sp. 40027 (Li et al. 2003), avoiding the need to screen for a Nal resistant recipient. Even if the procedures need customization for individual species before achieving acceptable efficiencies, conjugation with actinomycetes is simpler than DNA transformation methods, which require the production and regeneration of protoplasts. Moreover, *E. coli* is host to models of genetic regulation and is compatible with an evergrowing number of tools for genetic engineering. Therefore, other existing popular *E. coli* donor strains, such as S17-1 or SM10 could be modified by introducing the *dapA* mutation and inactivating further DNA methylation systems. Given that most seldom-studied or newly discovered actinobacterial strains lack adequate genetic tools, the use of a DAP-auxotroph *E. coli* donor is of interest to facilitate and accelerate molecular genetic studies on the highly heterogenous actinobacteria of ecological and industrial interest.

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Draft

580 Figure legends

581 **Fig. 1.** DAP deprivation is more efficient than Nal sensitivity to reduce *E. coli* viability.

582 A) Growth curves at 37 °C of *E. coli* ET12567 and its $\Delta dapA$ mutant in 50 ml LB
583 medium supplemented as indicated. The growth was estimated by optical density
584 measurements (OD_{600nm}). Data points are the means and SEM of three independent
585 experiments. B) Quantification of the CFUs corresponding to various time points
586 assessed in A. At each time point, 10 µl of serial dilutions of cultures were plated on LB
587 agar ± DAP and incubated at 37 °C for 18 h. C) Estimation of the membrane integrity of
588 *E. coli* ET12567 and its $\Delta dapA$ mutant at three time points assessed in A, using
589 propidium iodide staining and fluorescence microscopy. The results are the mean and
590 SEM of three independent experiments with 8 images per experiment per dye at each
591 time point.

592
593 **Fig. 2.** Assessment of the survival of *E. coli* ET12567 and its $\Delta dapA$ mutant following 5-
594 h mating assays and counterselection using either DAP deprivation or Nal sensitivity.
595 Mating partners were retrieved from the mating filters and plated on medium
596 supplemented as indicated. Cells were retrieved after 0, 2, 4, and 6 h of incubation at 30
597 °C and CFUs were enumerated by plating the appropriate dilutions on LB agar + Kan ±
598 DAP to counterselect the recipient strain. For each condition, the % of survival was
599 determined by dividing the CFUs at 2, 4, and 6 h by the CFUs at 0 h, an estimation of the
600 *E. coli* population right after conjugation. Results are the mean and SEM of four
601 independent experiments.

602

603 **Fig. 3.** DAP counterselection is suitable for intergeneric conjugation. A) Conjugative
604 transfer of pSET152 from *E. coli* ET12567/pUZ8002 and its $\Delta dapA$ mutant to *S.*
605 *coelicolor* A3(2) M145 in mating assays of 5 or 20 h. The mating partners were plated on
606 mannitol soya flour (MS) medium containing appropriate supplements for
607 counterselection. The frequency of exconjugants formation was calculated by dividing
608 the exconjugants CFUs (Apr^R) by the donors CFUs (Kan^R). For all the mating assays, the
609 donor *E. coli* ET12567/pUZ8002 ($\Delta dapA$) lacking pSET152 was used as a negative
610 control resulting in no Apr^R CFUs for all conditions. Results are the mean and SEM of
611 four independent experiments. Statistical analyses were performed using the two-tailed
612 Student's *t*-test to compare both counterselection techniques. B) Verification of the
613 integration of pSET152 into the chromosome of *S. coelicolor* exconjugants selected in 5-
614 h mating assays in A. Amplification of the transferred vector's selection marker (*acc(3)IV*
615 gene segment, 202 bp) and its chromosomal attachment sites (*attR*, 513 bp, and *attL*, 517
616 bp). Lanes: 1, *E. coli* ET12567/pSET152 $\Delta dapA$; 2, *S. coelicolor* A3(2) M145; 3-7, *S.*
617 *coelicolor* A3(2) M145/pSET152.
618

1 **Table 1.** Primers used in this study

Primer	Nucleotide sequence	Target	Fragment size (bp)
rpoDEcoli-F	5'-ATGGAGCAAAACCCGCAGTCAC-3'	<i>rpoD E.coli</i>	1820
rpoDEcoli-R	5'-AGCACTTCAGAACGGCTCGG-3'		
dapA-F	5'-ATGTTACGCGGAAGTATTGT-3'	<i>dapA E. coli</i>	879
dapA-R	5'-TTACAGCAAACCGGCAT-3'		
Apr-F	5'-GTTTCATGTGCAGCTCCATCAGCAA-3'	<i>acc(3)IV</i> gene	202
Apr-R	5'-ACGCTACGGAAGGAGCTGTG-3'	fragment	
Sco4178441-F	5'-CTGGACGCTGCCACTGCGGA-3'	<i>attR</i>	513
pSET-R	5'-GCCCCGGCGCTTCGCTGAAAT-3'		
pSET-F	5'-TCCAGACGTCCCGAAGGCGT-3'	<i>attL</i>	517
Sco4179035-R	5'-GAGGGAATGACCTCCGGGCT-3'		

2

3

4 **Table 2.** Time-course assay of cell viability.

Strain (medium)	Survival (%)	
	t = 210 min	t = 390 min
ET12567 (LB)	1 768 ± 229	10 376 ± 305
ET12567 (LB + Nal)	5.9 ± 0.6	1.7 ± 0.4
$\Delta dapA$ (LB + DAP + Nal)	6.2 ± 1.5	1.1 ± 0.05
$\Delta dapA$ (LB)	0.04 ± 0.007	0.004 ± 0.0004

5 Note: Viability was estimated as % of survival on LB agar supplemented or not with DAP,
6 obtained by dividing the number of CFUs at time 210 or 390 min by the number of CFUs
7 at time 0 during the growth curves assessed in Fig. 1A.

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Draft

Fig. 1. DAP deprivation is more efficient than Nal sensitivity to reduce *E. coli* viability.

Draft

Fig. 2. Assessment of the survival of *E. coli* ET12567 and its $\Delta dapA$ mutant following 5-h mating assays and counterselection using either DAP deprivation or Nal sensitivity.

Draft

1 **Fig. 3.** DAP counterselection is suitable for intergeneric conjugation.

2