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Research Article

The use of pAL5000 Replicon Vectors to Transform *Rhodococcus* and *Gordonia* Species

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ABSTRACT

pAL5000 based vectors, conventionally used to transform *Mycobacterium* species were used in this study to transform *Rhodococcus* and *Gordonia* species. Adequate transformation efficiencies were obtained ranging between $4.5x10^3$ – $2.8x10^6$. Additionally, a pAL5000 based vector with suicide function was generated. This vector was transformed into *Rhodococcus, Mycobacterium* and *Gordonia* species and was maintained as a stable construct.

Keywords: pAL5000, *Rhodococcus*, *Gordonia,* suicide vector.

INTRODUCTION

Nocardioformactinomycetes possess broad metabolic diversity. They are capable of the biosynthesis of an array of substrates and decomposition and utilization of harmful compounds. Their capacity to degrade recalcitrant compounds can be demonstrated by numerous studies. *R. rhodochrous* strains have been implicated in the degradation of crude oil, herbicides and pesticides (Harada et al., 2006; Sorkhoh et al., 1989). *R. opacus* can grow in the presence of aromatic carbons such as benzene, stryene and xylene (Na *et al.,* 2005). *R. erythropolis* has the ability to degrade toxic phenol (Vesely *et al*., 2003). *G. desulfuricans* NB4 has been implicated in the metabolism of organosulfur compounds (van Hamme *et al*., 2013). *G. rubripertincta* has been isolated from oil contaminated soil (Diaz-Ramirez *et al*., 2008). *M. smegmatis* is capable of breaking down triphenylmethane dyes (Guerra-Lopez *et al.,* 2007). In view of the benefits offered by this group of prokaryotes it is useful to construct suitable vectors for the investigation of these valuable traits. Cloning vectors have undoubtedly played a tremendous role in understanding prokaryotic genetics. They make possible the determination of gene function and the expression of gene products while contributing valuable information pertaining to metabolic pathways.

The *Mycobacterium* sp. cryptic plasmid, pAL5000 extracted from *M. for tuitum* is one of the most commonly used replicons in Mycobacterial derived vectors. The regions within this plasmid have been studied at length and the roles and interactions of the genes are well understood. pAL5000 replication genes share homology with the replicons of many other microbial species, suggesting an extended host range beyond *Mycobacteria*. Homologues of pAL5000 rep genes have thus far been found in *B. longum* (pMB1*), R. erythropolis* (pFAJ2600), *B. linens* (pBLA8), *N. gonorrheae* (pJD1) and *C. glutamicum* (pXZ10142) (Hatfull and Jacobs, 2000).

In this study we focused on finding an efficient vector transformation system for *Rhodococcus* sp. and *Gordonia* sp. strains which other studies have shown to be biotechnologically relevant. Consequently, we chose environmentally and industrially relevant strains: *R. opacus*, *R. erythropolis*, *R. rhodochrous*, *Gordonia* sp. NB4 and *G. rubripertincta* in order to investigate the effectiveness of using episomal Mycobacterial vectors to transform into these isolates. Additionally, we showed that the Mycobacterial replicon vector pOLYG, was capable of transforming all chosen isolates and thus was selected to generate a Gram positive suicide vector.

MATERIALS AND METHODS

Bacterial strains and vectors

Bacterial strains and vectors used are shown in Table 1 and Table 2.

Table1: Bacterial strains used in this study

Species	Strain	Characteristics	Source
Escherichia coli	MM294-4	*endA1, hsd R17, gyr A*, highly transformable	Genetics culture collection
Escherichia coli	λ lysogen	λ lysogen of MM294-4	Genetics culture collection
Rhodococcus erythropolis	SQ1	Highly transformable	Quan and Dabbs1993
Rhodococcus erythropolis	4277	Highly transformable	Genetics culture collection
Rhodococcus erythropolis	DSM 1069	Highly transformable	Deutsche Sammlung für Mikroorganismen und Zelkulturen
Rhodococcus opacus	HLPA1	Intermediate transformation efficiency	Genetics culture collection
Rhodococcus rhodochrous	RI8	Intermediate transformation efficiency	Genetics culture collection
Mycobacterium smegmatis	mc^2 155	Highly transformable	Genetics culture collection
Mycobacterium parafortuitum	490	Low transformation efficiency	Genetics culture collection
Gordonia rubripertincta	25593	Low transformation efficiency	Genetics culture collection
Gordonia desulfuricans	NB4	Environmental isolate, which metabolizes organosulfur compounds, low transformation efficiency	Van Hamme et al. (2013)
Gordonia desulfuricans	NB13	Environmental isolate which metabolizes organosulfur compounds, low transformation efficiency	van Hamme et al.,2013
Gordonia australis	A554	Low transformation efficiency	Genetics culture collection

Table 2: Vectors and its associated characteristics

Vector	Characteristics	Source
pEcoR251	*bla* resistance marker for Gram negatives, *Eco* RI endonuclease gene expression regulated by λ promoter, pMB1 replicon	Quan and Dabbs 1993
pDA71	*Rhodococcus* sp. replicon, *Eco* RI endonuclease gene expression regulated by λ promoter	Quan and Dabbs 1993
pNV18	pAL5000 ori, pMB1 replicon, blue-white selection, multiple cloning site	(Chiba et al., 2007)
pNV19	Same as pNV18 except the multiple cloning site is in the opposite orientation	(Chiba et al., 2007)
pOLYG	*E. coli-Mycobacterium* multicopy shuttle vector, Hygromycin resistance marker for Gram positive and Gram negatives	B. Gordhan and Dabbs,1994
pK4	*E. coli- Rhodococcus* shuttle vector, kanamycin resistance marker	Hashimoto et al., 1992
pCY104	*E. coli-Nocardia* shuttle vector, kanamycin, chloramphenicol, thiostrepton resistance markers	Yao et al., 1994

Electroporation of vectors into bacterial strains

Cultures were grown in LBSG (g/L): tryptone 10g, yeast extract 5g, NaCl 5g, sucrose 100g with appropriate glycine concentration overnight. These were transferred to Eppendorf tubes and the cells pelleted (13000 rpm; 2 min.) at 4°C. The cells were washed three times in sterile distilled water and resuspended in 1ml cold sterile distilled water.100µl of the culture was transferred into an Eppendorf tube with 500 ng of DNA. This was mixed by bubbling air through the mixture and transferred into a prechilled sterile electroporation cuvette. The electroporation parameters were set as follows: capacitance 25uF, voltage 2.5 kVand resistance 400Ω. The cuvette was electroporated and the time constant recorded after which LB (g/L) tryptone 10g, yeast extract 5g, NaCl 5g, was immediately added. A no DNA control was included. The cells were incubated on a shaker at 30°C for 2-5 h. Following the incubation, the cells were gently spread onto appropriate antibiotic plates. The vectors pNV18, pNV19, pK4 and pCY104 were selected on kanamycin plates (100 µg/ml). The vector pOLYG was selected on hygromycin plates (100 µg/ml). This was incubated at 30°C till growth was seen.

Construction of vector pCCC2

pCCC1 was formed by the ligation of pOLYG pEcoR251 at their respective *Bam* HI sites. Ligation of pOLYG pEcoR251 resulted in an 8615bp vector. To reduce the vector size, excess DNA (additional pMB1 replicon and *bla* determinant) was removed by digesting the vector with *SspI* and *Eco* RV and religating the vector once again, leading to the formation of pCCC2 (Figure 1).

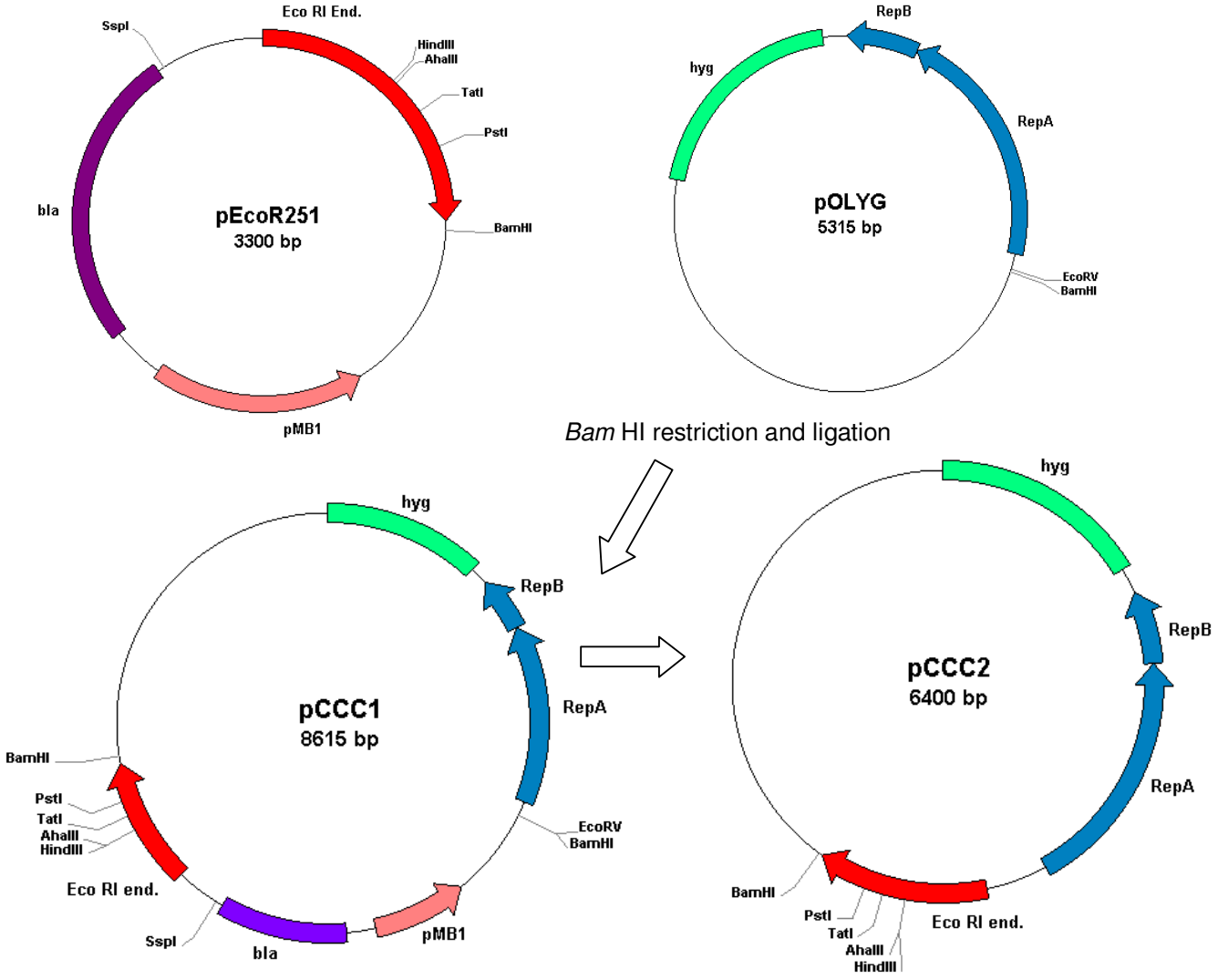

Figure1: Construction of vector pCCC2. Maps created with Plasm software.

RESULTS

The *Hind* III site present on pOLYG was filled in using the DNA polymerase I large Klenow fragment, effectively removing the site. Since a *Hind* III site is present on vector pEcoR251, this allowed a unique cloning site to be maintained once the suicide gene was introduced. Also the modification of this sequence did not introduce any unfavorable restriction sites (data not shown).

Vector host range

Host range of various vectors

The host range of vectors pNV18,pNV19,pOLYG,pCY104 and pK4 were tested in the bacterial species; *Rhodococcus*, *Mycobacteria* and *Gordonia* utilizing electroporation (Table3). The cells only control (no plasmid DNA) resulted in no colonies on any of the plates, suggesting all colonies on the plates carried vectors. To test this, forty potential transformants which grew on the antibiotic plates were selected and regrown in the appropriate antibiotic after which the vector was extracted and retransformed into *E. coli*. Afterwards, the vector was re-extracted from fifty selected *E. coli* colonies and digested (to linearize the plasmid) and examined on an agarose gel. The detection of a band of similar size to the original vector in relation to the molecular weight marker was used to verify the presence of the vector. In all cases vector was isolated from every transformant tested.

Table3: Host range of selected vectors

Species	Strain	pNV18	pNV19	pCY104	pK4	pOLYG
Rhodococcus erythropolis	SQ1	+	+	+	+	+
Rhodococcus erythropolis	ATCC 4277	+	+	+	+	+
Rhodococcus erythropolis	DSM 1069	+	+	+	+	+
Rhodococcus rhodochrous	RI8	+	+	+	+	+
Rhodococcus opacus	HL-PA1	+	+	+	+	+
Mycobacterium smegmatis	mc^2 155	+	+	+	+	+
Mycobacterium parafortuitum	490	+	+	-	-	+
Gordonia rubropertincta	ATCC 25593	+	+	+	+	+
Gordonia sp.	NB4	+	+	-	-	+
Gordonia sp.	NB13	+	+	-	-	+
Gordonia australis	A554	+	+	-	-	+

+ = transformants on plate, - = no transformants on plate

Transformation efficiency

The transformation efficiency of all vectors was determined using selected members of the species *Rhodococcus*, *Mycobacteria* and *Gordonia* (Table 4). These strains (*R. erythropolis SQ1* and *strain 1069, G. rubripertincta* and *M. smegmatis*) are suitable representatives of transformation efficiency from each genera. Cells were electroporated with 0.5µg of plasmid DNA and a serial dilution of cells was performed and plated. The transformation efficiency was then determined by calculating the number of colonies obtained per 1µg DNA. The resultant broad host range and high transformation efficiencies of pOLYG led to its selection for use in the construction of a suicide vector.

Table4: Number of transformants/µg vector DNA

Bacterial strains	pNV18	pNV19	pCY104	pK4	pOLYG
Rhodococcus erythropolis SQ1	$4.0x10^5$	$3.8x10^5$	$4.9x10^5$	$2.5x10^3$	$2.8x10^6$
Rhodococcus erythropolis 1069	$3.4x10^4$	$3.9x10^4$	$2.4x10^5$	$8.4x10^5$	$3.5x10^4$
*Gordoniarubropertincta*ATCC 25593	$4.8x10^4$	$4.9x10^4$	$1.8x10^2$	$1.6x10^1$	$4.5x10^3$
Mycobacterium smegmatis mc^2155	$6.8x10^3$	$7.6x10^3$	$5.1x10^3$	$4.3x10^3$	$2.1x10^4$

Construction of suicide vector pCCC2 and determination of structural stability

In order to generate a suicide vector suitable for use in *Rhodococcus* and *Gordonia* species, pOLYG was ligated to the *E. coli* suicide plasmid pEcoR251 generating pCCC1. pEcoR251 carries several unique restriction sites which includes *Aha*III, *Tat*I, *Pst*I and *Hind*III which are useful for cloning. pOLYG also harbours a *Hind*III site, thus in order to retain a unique *Hind*III site, one site was filled in using the DNA polymerase I large Klenow fragment, effectively removing the site. The modification of this sequence did not introduce any unfavorable restriction sites (data not shown). Subsequently, a restriction of pCCC1 removed the excess pMB1 replicon and *bla* gene creating pCCC2.

To ensure that pCCC2 was not rearranging upon transformation into Gram positive strains, we checked the structural stability of the vector as described by Mangan et al. (2005). Briefly, this was evaluated by extracting the vector from their Gram positive hosts, retransforming them into *E. coli*, re-extracting the vector and performing digestions. The size of the bands expected was calculated from the restriction map of the expected vector. pCCC2 results coincided with the estimations made, revealing no rearrangement or deletions (data not shown).

DISCUSSION

The focus of this study was to develop a positive selection vector with the suicide function reliant on the *Eco* RI endonuclease gene. Additionally, a broad host range vector was required which could be easily electroporated. The latter trait was tested by electroporating into members of the species *Rhodococcus*, *Mycobacterium* and *Gordonia*.

The original developers of pNV18 and pNV19 intended these shuttle vectors for use in *Nocardia* sp., since this genus has limited applicable cloning vectors (Chiba *et al*., 2007). *Rhodococcus* and *Gordonia* species face the same limitation although the number of vectors developed for these genera is on the increase (Dabbs *et al*., 1990; Bahn *et al.,* 2005; Matsui et al., 2007; Stanislauskiene et al., 2012). However, *Mycobacterium* sp. have many useful vectors. The exploration of pathogenesis in this genus has spurred the development of numerous transformation systems. Hatfull and Jacobs (2000) list 40 vectors developed for use in mycobacterial genetics, comprising cloning, expression and integrating plasmids.

As mentioned previously pAL5000 shares sequence similarity to replicons within unrelated bacteria. Thus, it can be presumed that this large number of vectors developed specifically for use in *Mycobacterium* sp. has the potential for applicability in other genera as well. With regard to *Gordonia* sp., recently constructed vectors based on pNC903 have been used to transform them. Vector pNC903 isolated from *R. rhodochrous* has an origin of replication similar to pAL5000 (Bahn *et al.,* 2005). Notably, it was transformable in 12 *Gordonia sp. with* efficiencies in the range of 10^2- 10^4 CFU/ /µg DNA (Arenskötter *et al.,* 2003).

Similarly, the cryptic plasmid pFAJ2600 isolated from *R. erythropolis* N186/21 showed similarity to pAL5000 *Rep A* and *Rep B*. The vector based on this plasmid was transformed in *R. erythropolis, R. fascians, R. rhodochrous* and *R. ruber* (DeMot *et al.,* 1997).

Encouragingly both pNV vectors and pOLYG were transformable in all strains tested. Comparative studies were conducted on two other shuttle vectors, namely pCY104, a Nocardial vector and pK4 a *Rhodococcus* replicon vector. Yao et al. (1994) publication regarding pCY104 mentioned a transformation efficiency of 8 x 10^4 CFU/µg DNA in *N. asteroids*. In this study the vector yielded an equally high efficiency in *R .erythropolis* of~$4.9x10^5$, though significantly poorer transformation in *Gordonia* and *Mycobacterium* sp. Both pK4 and pCY104 failed to transform *M. parafortuitum* and *Gordonia* sp. strains NB4 and NB13. It is possible that the replicons are either not recognized in these strains or electroporation parameters need to be adjusted to facilitate transformation. The low transformation efficiency of strains NB4 and NB13 with both pNV18 and pNV19 suggests the presence of a restriction modification system. In cases such as this the brief exposure of cells to a high temperature has proved to be effective temporarily in activating the restriction system (Engel, 1987).

The highest transformation of the pNV vectors and pOLYG was detected in *R. erythropolis* SQ1at an efficiency of $4.0x10^5$and $2.8x10^6$ transformants/µg DNA respectively. This is similar to experiments reported by Chiba et al. (2007). These researchers reported an efficiency of pNV18 and pNV19 in *N. farcinica* IFM10152 ranging between $2.4x10^5$ to$1.3x10^6$ CFU/µg DNA. Unexpectedly, these Mycobacterial replicon vectors were transformed at a lower efficiency in *M. smegmatis*, between $6.8x10^3$-$2.1x10^4$CFU/µg DNA. This most likely is due to the transformation conditions. Common protocols of *M. smegmatis* transformation utilize different electroporation solutions and variable electroporation parameters (Pelicic *et al.,*1997).

Studies have described that hygromycin carrying vectors have transformed Mycobacterial strains which were non-transformable with kanamycin vectors. Stolt and Stoker (1996) carried out investigations on vectors pYUB12 and pUH4 which differ only with respect to their antibiotic resistance genes; pYUB12 carried a kanamycin selective marker and pUH4 a hygromycin selective marker. These authors claimed that the lower stability of pYUB12 could be attributed to the kanamycin gene placing a greater burden on the cell than the hygromycin gene. Similarly, pNV18 and pOLYG are alike with the exception of their antibiotic resistance markers and multiple cloning sites. In this study the transformation of pOLYG yielded a higher efficiency than pNV18 in *M. smegmatis*. In this regard, there are many factors to consider in the transformation of pNV and pOLYG vectors. Transformation into *Gordoniasp*. was low. Arensk̈otter *et al.* (2003) described pNC9503 and pNC9501, *E. coli– Rhodococcus* shuttle vectors which were electroporated into *G. polyisoprenivorans.* Initial transformation led to approximately10^3transformants/µg DNA and 50% of these carried an identical 800 bp deletion.The transformation efficiency was improved to $4x10^5$ CFU/µg DNA and vector deletion prevented by applying heat shock. This was accomplished by incubation for 10 min. at 0^oC before and 6min. at 46^oC after electroporation. This suggests, it might be possible to improve *G. Rubropertincta* transformation efficiencies by inactivating the restriction system through heat shock (Arensk̈otter *et al.,* 2003).

Despite the efficiencies being low in *Mycobacterium* and *Gordonia* species these vectors are still sufficient for cloning purposes and can adequately be utilized for library construction. It should be noted that no optimization was attempted and thus these efficiencies can be improved upon.

In general vector DNA harvested from a Gram negative intended for use in a Gram positive leads to a reduced efficiency due to the presence of dam or dcm methylation. This can be improved upon by harvesting the DNA instead from a dam^-dcm^- strain or Gram positive related to the host strain. For example, Singer and Finnerty (1988) described pMVS301, which when harvested from a *Rhodococcus* strain led to a transformation efficiency of$1.9x10^5$CFU/µg DNA and lowered to $3.6x10^2$ CFU/ µgDNA when harvested from *E. coli*. A similar situation was described by Yao et al. (1994) who noted a 10^2-10^3drop in efficiency when harvesting DNA from *E. coli*.

From previously published articles the pNV vectors were transformed into several strains, namely *N. farcinica, N. asteroides, N. nova, N. cyriacigergica, Dietzia sp.* and *R. equi* (Chiba *et al.,* 2007; Mangan *et al.,* 2005; Szvetnik et al., 2010). This study has extended that host range to include *Rhodococcus* sp., *Gordonia* sp. and *Mycobacterium* sp.

Apart from the endonuclease gene, two other counter selectable suicide genes exist, namely *sacB* and *rpsL*. *Sac B*codes for levansucrase, which is responsible for the hydrolysis of sucrose and synthesis of levans. In the presence of sucrose its suicide function is initiated leading to an accumulation of levans and cell death (Hatfull and Jacobs, 2000).Thus this gene induces sucrose sensitivity. The gene *rpsL.* works in a similar fashion. It confers dominant streptomycin sensitivity in streptomycin resistant strains (Hosted and Baltz, 1999). In pEco R251the *Eco* RI endonuclease gene is controlled by the P_R promoter. When this vector is transformed into a non-lysogen strain with an intact endonuclease gene, its expression leads to DNA digestion and resultantly killing of the cell. To overcome this, DNA can be introduced into a unique site of the endonuclease gene, eliminating gene function and preventing cell death. In this regard only cells carrying DNA inserts will survive, revealing the practical use of selection vectors (Zabeau and Stanley,1982; Dabbs *et al.,*1990; Chengalroyen and Dabbs, 2012; Chengalroyen and Dabbs, 2013).Vector pDA71is a well recognized *Rhodococcus* suicide vector based on the *Eco* RI gene. This vector and its predecessors have been used to clone genes involved in the degradation of azodyes, rifampicin in activation and pigment synthesis (Dabbs, 1998). From these studies it is clear that a positive selection feature is useful, offering many advantages.

Although the vector pCCC2 needs to be improved upon it carries useful features in that it can be transformed using electroporation, a convenient and reproducible procedure when compared to PEG mediated protoplast transformation. Moreover, it possesses a potentially wide host range and was transformed into 3 *Rhodococcus* sp., 2 *Mycobacteria* sp. and 3 *Gordonia* sp. The vector pCCC2 is structurally stable in Gram positive species and upon improvement holds potential as a useful cloning vector.

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