J Ind Microbiol Biotechnol (2010) 37:1097–1103
DOI 10.1007/s10295-010-0807-5

SHORT COMMUNICATION

Methods for the genetic manipulation of *Nonomuraea* sp. ATCC 39727

Giorgia Letizia Marcone · Lucy Foulston · Elisa Binda · Flavia Marinelli · Mervyn Bibb · Fabrizio Beltrametti

Received: 14 June 2010 / Accepted: 4 August 2010 / Published online: 18 August 2010

Abstract *Nonomuraea* sp. ATCC 39727 belongs to the *Streptosporangiaceae* family of filamentous actinomycetes. This microorganism produces the teicoplanin-like glycopeptide A40926, which is the starting material for the synthesis of the second-generation glycopeptide dalbavancin. Notwithstanding the strain's pharmaceutical relevance, the lack or poor efficiency of genetic tools to manipulate *Nonomuraea* sp. ATCC 39727 has hampered strain and product improvement. Here we report the development of gene transfer systems based on protoplast transformation and intergeneric conjugation from *Escherichia coli*. Efficiency of transformation and conjugation, followed by site specific or homologous recombination with the *Nonomuraea* sp. genome, were determined using the integrative plasmid pSET152 (5.7 kb), and the Supercos1 derivative cosmid A40ΔY (30 kb). To our knowledge, this is the first report of the transformation of protoplasts of *Nonomuraea* sp. ATCC 39727, even though the improved procedure for intergeneric conjugation makes it the method of choice for introducing large segments of DNA into *Nonomuraea* sp. ATCC 39727.

Keywords Actinomycetes · Antibiotics · *Nonomuraea* · Conjugation · Protoplasts

G. L. Marcone (✉) · E. Binda · F. Marinelli
Dipartimento di Biotecnologie e Scienze Molecolari, Università degli Studi dell'Insubria, via J. H. Dunant 3, Varese 21100, Italy
e-mail: giorgia.marcone@uninsubria.it

G. L. Marcone · E. Binda · F. Marinelli
Centro Interuniversitario di Ricerca in Biotecnologie Proteiche "The Protein Factory", Politecnico di Milano and Università degli Studi dell'Insubria, Varese, Italy

L. Foulston · M. Bibb
John Innes Centre, Norwich Research Park, Colney Lane, Norwich NR4 7UH, UK

F. Beltrametti
Actygea S.r.l., via R. Lepetit 34, 21040 Gerenzano (VA), Italy

Introduction

The so-called rare or uncommon actinomycetes include filamentous actinomycetes other than *Streptomyces* that are difficult to isolate, cultivate and genetically manipulate [1]. Some of these difficult-to-handle microorganisms produce medically valuable antibiotics. However, the study and cost-effective exploitation of uncommon actinomycetes, including both strain and product improvement, have been hampered by a lack of genetic tools. The presence of potent restriction-modification systems and the absence of efficient transformation protocols have particularly hindered the development of genetic systems in many actinomycetes [2].

While procedures for protoplast transformation were successfully developed for *Streptomyces* spp. [3], they are not generally applicable to other actinomycetes [4–7]. Transformation by electroporation has been reported, but it is of low efficiency and of limited application [2]. We recently developed a system for protoplast preparation, fusion and regeneration for uncommon actinomycetes producing metabolites of industrial and medical interest [8, 9]. There has also been considerable interest in the use of intergeneric conjugation as a means of transferring single-stranded DNA, which evades restriction barriers, into actinomycetes, even if procedures have to be customised for individual species before achieving acceptable conjugation efficiencies [4, 10]. Here we describe the development of

methods for the manipulation of *Nonomuraea* sp. ATCC 39727. This actinomycete belongs to the *Streptosporangiaceae* family and produces the glycopeptide antibiotic A40926, the precursor of the semi-synthetic derivative dalbavancin, a second-generation glycopeptide currently in clinical development [11, 12]. Methods for the genetic manipulation of the A40926-producing strain will allow knowledge-based improvements in antibiotic productivity and the application of combinatorial biosynthesis to develop novel, potentially improved A40926 derivatives.

Materials and methods

Bacterial strains, vectors and recombinant DNA methods

Nonomuraea sp. ATCC 39727 was used as the recipient strain throughout this study. *E. coli* strain DH5α (Promega) was used as general host for cloning. For transformation and conjugation experiments, plasmids were prepared and maintained as follows. The site-specific integration vector pSET152 (5.7 kb) was extracted from *E. coli* strains S17-1 [13] and ET12567 [14] using the Qiagen Midi DNA extraction kit (Qiagen). The integrative plasmid pSET152 contains ΦC31 *int*, *attP* and *oriT* of RK2, as well as an apramycin resistance gene for selection in actinomycetes and *E. coli* [15]. pSET152 is not able to replicate autonomously in actinomycetes, and can be maintained in recipient strains only by integration at a chromosomal *attB* site. The Supercosl derivative cosmid A40ΔY (30 kb) [16] contains the T3, T7 and SV40 promoters, the pUC origin of replication, and ampicillin and neomycin resistance genes for selection in *E. coli* and some actinomycetes, respectively. Since cosmid A40ΔY lacks an origin of replication for actinomycetes, its maintenance in *Nonomuraea* sp. ATCC 39727 requires homologous recombination with the chromosome mediated through the inserted 22 kb segment of *Nonomuraea* sp. ATCC 39727 DNA. Cosmid extraction was performed by alkaline lysis following the method reported by Kieser [3]. The methylation-deficient *E. coli* strain ET12567 (*dam-13*::*Tn9*, *dcm-6*, *hsdM*, *hsdS*) containing the non-transmissible helper plasmid pUZ8002 was used as the donor strain in biparental intergeneric conjugations [17].

Media and culture conditions

Nonomuraea sp. ATCC 39727 was maintained as a lyophilised master cell bank (MCB). A working cell bank (WCB) was prepared from a first generation slant originating from the MCB as previously described [8, 18]. Mycelium for protoplast preparation was grown in 100 ml VM liquid medium [8] in 500 ml Erlenmeyer baffled flasks incubated at 28°C and 200 rpm. Growth was measured as dry weight as previously described [18]. Conditions for growth and protoplast regeneration of *Nonomuraea* sp. ATCC 39727 were essentially as described in Marcone et al. [8]. In brief, agar medium for growth of *Nonomuraea* sp. ATCC 39727 was V0.1 medium, and the hypertonic medium used for protoplast regeneration was M3 overlaid with VMS0.1 [8]. VSP [8] was used to grow *Nonomuraea* sp. ATCC 39727 to prepare biomass for intergeneric conjugation. Agar media assessed for conjugation were V0.1, V0.1 supplemented with 10 mM $MgCl_2$, and M3. For the extraction of chromosomal DNA from the transformants/exconjugants of *Nonomuraea* sp. ATCC 39727, antibiotic-resistant clones were inoculated into 10 ml VSP medium containing 100 μg/ml apramycin. Cultures were grown for three days at 30°C, then a further 10 ml of the same medium were added to each flask. Growth was continued for an additional 24 h. Samples were collected by centrifugation and DNA extraction was performed according to the Kirby procedure [3]. *E. coli* strains were grown at 37°C for 12 h on a rotary shaker (180 rpm) in Luria Bertani medium (LB, Difco) supplemented with 50 μg/ml apramycin, 25 μg/ml chloramphenicol, 100 μg/ml ampicillin and 50 μg/ml kanamycin as required.

Preparation, regeneration and protoplast transformation

Protoplasts of *Nonomuraea* sp. ATCC 39727 were prepared and regenerated as already described [8]. For transformation, protoplasts were concentrated to 2–4 × 10^9 in 100 μl of P medium and different amounts of plasmid or cosmid DNA (in TE buffer) added in a maximum volume of 40 μl. 1 ml of 25% polyethylene glycol (PEG) 1000 or Lipofectamine™ 2000 (Invitrogen) in P medium was added to the protoplast/DNA mixture, followed by incubation for a maximum of two min at room temperature. The transformation mix was centrifuged in a table-top centrifuge for 1 min and immediately suspended in 700 μl of P medium. 0.2 ml of the transformation mixture was plated on each M3 plate and overlaid with 4 ml of melted pre-cooled VMS0.1 medium. Protoplasts were allowed to regenerate at 30°C for one to three days and were then overlaid with 4–8 ml of VMS0.1 medium supplemented with apramycin at 50 μg/ml. Plates were incubated for an additional 10–15 days until resistant colonies appeared. Resistant colonies were then replicated with a toothpick onto fresh V0.1 medium plates supplemented with 100 μg/ml apramycin. After 5–6 days of growth, colonies were inoculated in liquid medium for DNA extraction as reported above.

Conjugation

Intergeneric conjugation was achieved as suggested by Kieser et al. [3] with modification. In brief, a culture of the donor *E. coli* ET12567/pUZ8002 containing the selected plasmid was grown in 10 ml LB supplemented with 50 μg/ml apramycin, 25 μg/ml chloramphenicol and 50 μg/ml kanamycin to an OD_{600} of 0.4. Cells were collected by centrifugation, washed twice with an equal volume of LB, and resuspended in 1 ml of LB. *Nonomuraea* sp. ATCC 39727 mycelium was prepared as follows. The strain was grown in 20 ml VSP medium in a 100 ml baffled flask containing 2 ml sterile glass beads (diameter 4 mm) and incubated at 28°C on a rotary shaker at 200 rpm to exponential phase (about 24 h). Mycelium was collected and washed twice with ice-cold glycerol 20%. After centrifugation, the pellet was suspended in half the initial volume of glycerol 20% and stored at −80°C. For conjugation, 0.5 ml *E. coli* donor cells (ca. 1×10^7 cells/ml) were added to the mycelium (0.5 ml) and the mixtures were spread on V0.1 plates containing 10 mM $MgCl_2$. The conjugation plates were incubated for 20–22 h at 30°C, and were then overlaid with 1 ml water containing 1 mg nalidixic acid and 1.25 mg apramycin. The plates were incubated at 30°C for 10–15 days. To confirm the chromosomal integration of the pSET152 or of the A40ΔY cassette, the exconjugants were analyzed by colony-PCR and sequencing of PCR products.

Colony PCR for *Nonomuraea* sp. ATCC 39727

Single colonies (streaked from ex-conjugants) were patched onto V0.1 medium. Plates were incubated at 30°C for 3–5 days. Mycelium was scraped from the plates using a sterile toothpick and was introduced into 50 μl 100% DMSO (Sigma-Aldrich) in a 1.5 ml tube containing two sterile glass beads. The tube was shaken vigorously for 1–2 h and then centrifuged briefly to pellet cell debris. 2.5 μl of the supernatant was used instead of the 2.5 μl of DMSO in a standard colony PCR. Control DNA samples were diluted 1 μl into 1.5 μl DMSO and were used in the same way. One initial step of 10 min at 95°C was included in the PCR program to ensure the complete cell lysis. The PCR was performed for 30 cycles as follows: 95°C for 45 s, 56°C for 30 s and 72°C for 1 min.

pSET152F and pSET152R were used as primers to amplify an internal fragment of pSET152 [19]. Primers C31F (5′-CTGCTGCACCAGCCACTC-3′) and C31R (5′-GATGGGCGAGGTCGACTAC), designed from the sequence surrounding the ΦC31 *attB* site of *Nonomuraea* sp. (GenBank: AJ564722.1), and LF45F and LF45R [19], were used to amplify across the predicted junctions formed by the integration of pSET152 at the *Nonomuraea* sp. ΦC31 *attB* site, thus confirming the integration of the plasmid in the *Nonomuraea* sp. chromosome.

Results and discussion

Protoplast transformation with pSET152 or cosmid A40ΔY

Protoplast transformation was initially performed with the integrative plasmid pSET152 (5.7 kb) that contains the bacteriophage ΦC31 *att/int* site-specific integration system. The *attB* integration site of *Nonomuraea* sp. ATCC 39727 has been previously sequenced and found to be homologous to the corresponding sequences found in *Streptomyces* [20], as well as to the recently reported *attB* site in *Actinoplanes teichomyceticus* [10]. *Nonomuraea* sp. ATCC 39727 was reported to be resistant to antibiotics such as chloramphenicol, erythromycin and hygromycin, which are commonly used for selection [20]. In contrast, the minimal inhibitory concentration of apramycin for *Nonomuraea* sp. ATCC 39727 on agar medium is less than 1 μg/ml, permitting the use of the apramycin resistance gene of pSET152 as selectable marker.

Preliminary experiments indicated that transformation was much more efficient with plasmid DNA extracted from the non-methylating *E. coli* ET12567 than from the methylating *E. coli* S17-1 (data not shown), suggesting that *Nonomuraea* sp. ATCC 39727, like many other actinomycetes, contains a methylated-DNA-dependent restriction system [10, 15]. Protoplasts were prepared from mycelium grown to both the exponential (ca. 20 h) and stationary phases of growth (ca. 48 h): nearly 50% of the protoplasts regenerated from mycelium harvested during active growth while 30% regenerated from stationary phase cells. PEG 1000 or Lipofectamine™ 2000 was used to facilitate transformation (the latter is often employed to transfect eukaryote cells [21]). The results are shown in Table 1. To confirm that the apramycin resistant colonies were transformants rather than spontaneous apramycin resistant mutants, PCR amplification of the *aac(3)IV* gene of pSET152 was performed on genomic DNA from 10 randomly selected apramycin resistant clones. All of the selected clones yielded a DNA fragment corresponding in size to the fragment amplified from the control pSET152 (data not shown). *Nonomuraea* sp. ATCC 39727 genomic DNA was used as the negative control. Since pSET152 cannot replicate autonomously in actinomycetes, it is assumed that the plasmid had integrated stably at the ΦC31 *attB* site (see Conjugal transfer of pSET152 below).

As expected from the regeneration efficiencies, protoplasts made from the exponential phase culture gave

Table 1 pSET152 transformation of *Nonomuraea* sp. ATCC 39727

μg DNA	PEG 1000		LIPOFECTAMINE™	
	Colonies Apra^R per ml[a]	Efficiency of transformation[b]	Colonies Apra^R per ml[a]	Efficiency of transformation[b]
Exponential phase				
0	0	0	0	0
0.1	234	2,400	42	431
0.5	nd	nd	1,048	2,150
1	1,499	1,538	nd	nd
2	797	401	7,305	3,675
8	663	83	519	65
Stationary phase				
0	0	0	0	0
0.1	63	646	1	13
0.5	nd	nd	5	10
1	400	410	nd	nd
2	334	168	8	4
8	523	65	29	4

Values represent the average of three independent experiments where the SD was less than 5%

[a] 1 ml contained 10^8 protoplasts before transformation. Apra^R colonies were selected on 50 μg/ml of apramycin

[b] Calculated as number of apramycin resistant colonies per μg of DNA used in the transformation

markedly higher transformation frequencies than those prepared from stationary phase mycelium. While there was no consistent difference in transformation efficiency between PEG 1000 and Lipofectamine™ 2000, transformation in the presence of Lipofectamine™ 2000 gave optimal results when using 2 μg of plasmid DNA (3.7×10^3 transformants per μg of plasmid DNA; Table 1). This presumably reflects that Lipofectamine™ 2000 better complexes higher concentrations of nucleic acids, allowing the transformation with higher DNA concentrations in comparison to PEG [21]. The highest efficiencies obtained here are markedly lower than those reported for *Streptomyces* spp. (10^6 transformants per μg of plasmid DNA) [3].

Protoplast transformation was then performed with the Supercos-1 cosmid derivative A40ΔY designed to inactivate the *vanY* gene putatively involved in self-resistance to A40926 in *Nonomuraea* sp. ATCC 39727 [16]. A40ΔY contains a 22 kb segment of the *Nonomuraea* sp. ATCC 39727 chromosome and does not replicate in *Nonomuraea*. Thus, stable transformants can only be obtained by homologous recombination.

Cosmid A40ΔY was extracted from the non-methylating *E. coli* ET12567 and used to transform protoplasts following the protocol described above for transformation with pSET152. Different protoplast and DNA concentrations were used. However, no transformants were obtained. This likely reflects the larger size of the plasmid used and/or to the different method of integration into the recipient genome. Denaturation by alkali treatment of the cosmid just before transformation, as described in [3], also failed to yield transformants.

Conjugal transfer of pSET152

In order to assess whether we could transfer large segments of DNA into *Nonomuraea* sp. ATCC 39727, we first developed a conjugation protocol using the small integrative plasmid pSET152. Initially, intergeneric conjugation between *Nonomuraea* sp. ATCC 39727 and *E. coli* ET12567/pUZ8002 containing pSET152 was attempted by adapting the protocol used with *Streptomyces* spores [3]; since conditions for sporulation of *Nonomuraea* sp. ATCC 39727 have not been determined, mycelium was used as the recipient.

As reported for protoplast production [8], it was first necessary to determine conditions that gave dispersed growth of *Nonomuraea* sp. ATCC 39727 in liquid culture. SFM, ISP4 and RARE3 [3, 20] liquid media all yielded compact pellets of mycelium not suitable for conjugation. Only VSP medium (with glass beads added to the flasks) gave dispersed growth. Furthermore, we observed that freezing of *Nonomuraea* sp. ATCC 39727 mycelium before conjugation enhanced dispersion. The agar medium used to select exconjugants can greatly influence the efficiency of conjugation in actinomycetes [10, 22]. Therefore, six representative media were tested. We selected medium V0.1 (routinely used to grow *Nonomuraea* sp. ATCC 39727), M3 (previouly optimized for protoplast regeneration [8]), BTT (already reported in another conjugation protocol [20] for *Nonomuraea* sp. ATCC 39727), and MS (suitable for streptomycetes and more recently for the non-*Streptomyces* actinomycete *Actinoplanes teichomyceticus* [10]). To increase the conjugation efficiency of *Streptomyces* strains, 10 mM $MgCl_2$ is generally added to agar

Table 2 Effect of media on intergeneric conjugation with pSET152

Medium	Vector	Colonies Apra^{R} [a]	Frequency of Apra^{R} [b]
MS	pSET152	0	0
	A40ΔY	nd	nd
MS + 10 mM $MgCl_2$	pSET152	0	0
	A40ΔY	nd	nd
BTT	pSET152	0	0
	A40ΔY	nd	nd
V0.1	pSET152	60	1.2×10^{-5}
	A40ΔY	nd	nd
V0.1 + 10 mM $MgCl_2$	pSET152	4,040	0.8×10^{-3}
	A40ΔY	55	1.1×10^{-5}
M3	pSET152	2,120	4.2×10^{-4}
	A40ΔY	nd	nd

Conjugation with A40ΔY was performed only on the best medium for pSET152. Values represent the average from three independent experiments. 500 µl of an *E. coli* culture grown up to 0.4 OD_{600} (1×10^7 cells/ml) and 0.5×10^7 CFUs of *Nonomuraea* sp. ATCC 39727 were used in each conjugation

[a] Apra^{R} colonies are selected as resistant to 50 µg/ml of apramycin

[b] Calculated as number of apramycin resistant colonies per number of CFUs of *Nonomuraea* sp. ATCC 39727 used in the conjugation

media [3], although the function of Mg^{2+} has not been investigated in detail. Therefore, 10 mM $MgCl_2$ was added to medium V0.1 and MS (M3 already contained 10 mM $MgCl_2$). The results reported in Table 2 show that the frequency of pSET152 conjugation (calculated as the number of apramycin resistant colonies per *Nonomuraea* sp. ATCC 39727 CFU determined from the biomass used for conjugation) varies widely depending on the agar medium used. With MS (the most commonly used media for streptomycetes [3, 22] and for *Actinoplanes teichomyceticus* [10]), we did not obtain any exconjugants, whereas in media specifically developed for *Nonomuraea* sp. ATCC 39727 [8], we had the highest number of resistant clones. This suggests that the species-specific fine-tuning of medium composition is crucial in conjugation as well as in protoplast regeneration [8]. The addition of $MgCl_2$ plays a positive role when added to V0.1, increasing conjugation efficiency several fold and consistent with previous observations for *Actinoplanes teichomyceticus* [10]. The best medium (V0.1 supplemented with 10 mM $MgCl_2$) gave 4,040 colonies resistant to 50 µg/ml of apramycin from a conjugation mixture of 500 µl of *Nonomuraea* sp. ATCC 39727 (1×10^7 CFU/ml) and 500 µl of *E. coli* ET12567/pUZ8002 (OD_{600} of ca. 0.4, 1×10^7 cells/ml). One hundred randomly chosen colonies resistant to apramycin were analysed by PCR. Figure 1 shows amplification of a DNA fragment corresponding to the *aac(3)IV* gene in four representative clones, indicating that they are

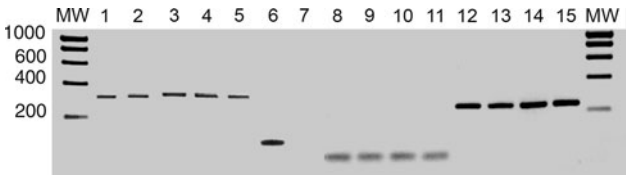

Fig. 1 PCR analysis of genomic DNA from 4 putative *Nonomuraea* sp. pSET152 exconjugants (1–4 and 8–15) compared to *Nonomuraea* sp. ATCC 39727 wild type genomic DNA (7) and pSET152 DNA (5–6). *Lanes 1–5* show the PCR products obtained with primers pSET152F and pSET152R, *lane 6* the PCR product of primers LF45F and LF45R, *lanes 7–11* the PCR products of primers LF45F and C31R, and *lanes 12–15* the PCR products of primers C31F and LF45R. The marker (Molecular Weight, *MW*) was a 1 kb ladder (NEB). The following expected band sizes were observed (based on the sequence information): 319 bp using pSET152F and pSET152R, 74 bp using LF45F and C31R, and 255 bp using C31F and LF45R, confirming integration of pSET152 at the ΦC31 *attB*

true exconjugants and not spontaneous resistant mutants. This was confirmed by the analysis of the integration site of pSET152 (Fig. 1).

The frequency of 10^{-3} exconjugants per recipient *Nonomuraea* sp. ATCC 39727 CFU is comparable with data reported for the same vector in other actinomycetes, such as *Actinoplanes teichomyceticus* ($\sim 10^{-3}$) [10] and *Streptomyces pristinaespiralis* ($\sim 10^{-3}$) [22]. Use of the ΦC31 *att/int* system in *Streptomyces lividans* is reported to give a higher frequency (8×10^{-2}) [3, 10].

Conjugal transfer of cosmid A40ΔY

The protocol developed for the conjugal transfer of pSET152 was used to introduce cosmid A40ΔY from the non-methylating *E. coli* ET12567/pUZ8002 into *Nonomuraea* sp. ATCC 39727. In these conditions (medium V0.1 supplemented with 10 mM $MgCl_2$; 500 µl of *Nonomuraea* sp. (1×10^7 CFU/ml) and 500 µl of *E. coli* ET12567/pUZ8002 (1×10^7 CFU/ml)), 55 colonies resistant to 50 µg/ml of apramycin were obtained, i.e. 1.1×10^{-5} exconjugants per *Nonomuraea* sp. ATCC 39727 CFU. Table 2 shows that there is a reduction in conjugation efficiency of about one hundred fold in comparison to pSET152, likely due to the larger size of the transferred plasmid and/or the method of chromosomal integration (i.e. site specific integration vs homologous recombination).

From the PCR/agarose gel electrophoresis analysis of the 55 apramycin resistant colonies (as described in "Materials and methods"), 37 showed a PCR amplification profile consistent with integration of cosmid A40ΔY into the *Nonomuraea* sp. ATCC 39727 genome by a single homologous recombination event, while 18 colonies showed a PCR amplification profile consistent with a double cross-over event that replaced *vanY* with the apramycin resistance cassette (for details see [23]). Figure 2

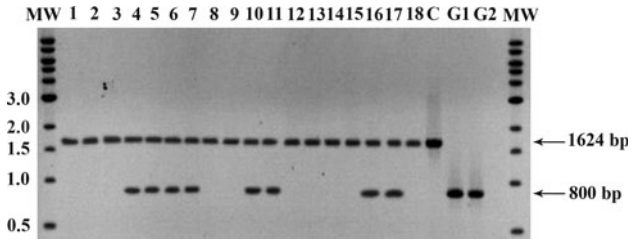

Fig. 2 Amplification of the inactivation cassette (containing *aac(3)IV* flanked by two *oriT* and FLP sites) in apramycin resistant clones from conjugation experiments with cosmid A40ΔY. Cosmid A40ΔY DNA was used as positive control (*C*), while genomic DNA from *Nonomuraea* sp. ATCC 39727 wild-type was used as negative control (*G1* and *G2*). 1 kb NEB: molecular weight markers. Two bands (1,624 and 800 bp), indicating that a single homologous recombination event occurred following conjugation, are present in eight clones tested, while 10 clones show a single band (1,624 bp) corresponding to two homologous recombination events (i.e. a double cross-over)

shows the pattern of bands resulting from colony-PCR performed from a sample of 18 exconjugants. Cosmid A40ΔY and genomic DNA from *Nonomuraea* sp. ATCC 39727 were used as positive and negative controls, respectively.

Minimal inhibitory concentration (MICs) of A40926 were determined for five of the 18 positive clones: all gave a MIC value of 2 μg/ml for A40926 rather than the 4 μg/ml of the wild-type strain, thus confirming inactivation of *vanY* and its involvement in self-resistance to A40926 in *Nonomuraea* sp. [16].

Conclusions

Two gene transfer systems were developed and optimized for *Nonomuraea* sp. ATCC 39727. The first one is based on protoplast production, transformation and regeneration, and requires de-methylated DNA. The second is based on *E. coli* ET12567/pUZ8002-*Nonomuraea* sp. ATCC 39727 intergeneric conjugation and was used successfully with both a 5.7 kb plasmid DNA and a 30 kb cosmid.

With the site-specific integrative plasmid pSET152, protoplasts were transformed with an efficiency of 1.5×10^3 per μg of plasmid DNA and 1.5×10^{-5} transformants per regenerating protoplast, whereas conjugation efficiency was estimated to be 10^{-3} per *Nonomuraea* sp. ATCC 39727 CFU.

With cosmid A40ΔY, which requires homologous recombination, the efficiency of conjugation was 1.1×10^{-5} per *Nonomuraea* sp. ATCC 39727 CFU, and was sufficient to isolate both single and double homologous recombination events.

The gene transfer systems developed here for *Nonomuraea* sp. ATCC 39727, combined with the recently reported use of *Nonomuraea* sp. as a host for the heterologous expression of an antibiotic biosynthetic gene cluster [19], further demonstrate the ability to genetically manipulate this previously relatively intractable actinomycete. The methods described here can now be used to better understand A40926 biosynthesis and self-resistance, and subsequently to manipulate the producing organism to enhance productivity and potentially produce A40926 derivatives with improved characteristics.

Acknowledgments This work was supported by FAR 2007–2008–2009 to F.M. and by MIUR fellowship to G.L.M. We thank also the support from Consorzio Interuniversitario per le Biotecnologie (CIB) and Progetto Cariplo: Promuovere Capitale Umano d'Eccellenza, for the grants to G.L.M. and E.B. L.F. and M.B. were funded by grants from the Biotechnology and Biological Sciences Research Council, U.K.

References

1. Lazzarini A, Cavaletti L, Toppo G, Marinelli F (2001) Rare genera of actinomycetes as potential producers of new antibiotics. Antonie Van Leeuwenhoek 79:399–405
2. Baltz RH (1998) Genetic manipulation of antibiotic-producing *Streptomyces*. Trends Microbiol 6:76–83
3. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Practical Streptomyces Genetics. The John Innes Foundation, Norwich
4. Stegmann E, Pelzer S, Wilken K, Wohlleben W (2001) Development of three different gene cloning systems for genetic investigation of the new species *Amycolatopsis japonicum* MG417-CF17, the ethylenediaminedisuccinic acid producer. J Biotechnol 92:195–204
5. Matsushima P, McHenney MA, Baltz RH (1987) Efficient transformation of *Amycolatopsis orientalis* (*Nocardia orientalis*) protoplasts by *Streptomyces* plasmids. J Bacteriol 169:2298–2300
6. Rajnisz A, Solecka J, Kurzatkowski W (2005) Properties of *Saccharopolyspora erythraea* strains after protoplast regeneration. Folia Microbiol (Praha) 50:13–18
7. Palleroni NJ (1983) Genetic recombination in *Actinoplanes brasiliensis* by protoplast fusion. Appl Environ Microbiol 45:1865–1869
8. Marcone GL, Carrano L, Marinelli F, Beltrametti F (2010) Protoplast preparation and reversion to the normal filamentous growth in antibiotic-producing uncommon actinomycetes. J Antibiot (Tokyo) 63:83–88
9. Beltrametti F, Barucco D, Rossi R, Selva E, Marinelli F (2007) Protoplast fusion and gene recombination in the uncommon actinomycete *Planobispora rosea* producing GE2270. J Antibiot (Tokyo) 60:447–454
10. Ha HS, Hwang YI, Choi SU (2008) Application of conjugation using *phiC31* att/int system for *Actinoplanes teichomyceticus*, a producer of teicoplanin. Biotechnol Lett 30:1233–1238
11. Goldstein BP, Selva E, Gastaldo L, Berti M, Pallanza R, Ripamonti F, Ferrari P, Denaro M, Arioli V, Cassani G (1987) A40926, a new glycopeptide antibiotic with anti-*Neisseria* activity. Antimicrob Agents Chemother 31:1961–1966
12. Cornaglia G, Rossolini GM (2009) Forthcoming therapeutic perspectives for infections due to multidrug-resistant Gram-positive pathogens. Clin Microbiol Infect 15:218–223
13. Simon B, Priefer UB, Puhler A (1983) A broad host range mobilization system for in vivo genetic engineering: Transposon

mutagenesis in Gram-negative bacteria. Biotechnology 1:784–791
14. MacNeil DJ, Gewain KM, Ruby CL, Dezeny G, Gibbons PH, MacNeil T (1992) Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. Gene 111:61–68
15. Bierman M, Logan R, O'Brien K, Seno ET, Rao RN, Schoner BE (1992) Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene 116:43–49
16. Marcone GL, Beltrametti F, Binda E, Carrano L, Foulston L, Hesketh A, Bibb M, Marinelli F (2010) Novel mechanism of glycopeptide resistance in the A40926 producer *Nonomuraea* sp. ATCC 39727. Antimicrob Agents Chemother 54:2465–2472
17. Paget MS, Leibovitz E, Buttner MJ (1999) A putative two-component signal transduction system regulates sigmaE, a sigma factor required for normal cell wall integrity in *Streptomyces coelicolor* A3(2). Mol Microbiol 33:97–107
18. Beltrametti F, Jovetic S, Feroggio M, Gastaldo L, Selva E, Marinelli F (2004) Valine influences production and complex composition of glycopeptide antibiotic A40926 in fermentations of *Nonomuraea* sp. ATCC 39727. J Antibiot (Tokyo) 57:37–44
19. Foulston LC, Bibb MJ (2010) Microbisporicin gene cluster reveals unusual features of lantibiotic biosynthesis in actinomycetes. Proc Natl Acad Sci USA (in press)
20. Stinchi S, Azimonti S, Donadio S, Sosio M (2003) A gene transfer system for the glycopeptide producer *Nonomuraea* sp. ATCC 39727. FEMS Microbiol Lett 225:53–57
21. Dalby B, Cates S, Harris A, Ohki EC, Tilkins ML, Price PJ, Ciccarone VC (2000) Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. Methods 2004(33):95–103
22. Jin Z, Jin X, Jin Q (2010) Conjugal transferring of resistance gene *ptr* for improvement of pristinamycin-producing *Streptomyces pristinaespiralis*. Appl Biochem Biotechnol 160:1853–1864
23. Gust B, Challis GL, Fowler K, Kieser T, Chater KF (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci USA 100:1541–1546