TRANSFORMATION OF *STREPTOMYCES ERYTHRAEUS*

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Streptomyces erythraeus strains were transformed efficiently with six different plasmid DNA vectors by a protocol that uses 0.2 mM Ca^{2+}, 5 mM Mg^{2+} and 10% DMSO to enhance the stability of protoplasts to storage at −80°C and their transformability at 30°C. The primary thiostrepton-resistant ($Thio^r$) transformants for most vectors were unstable even when grown selectively. This instability did not appear to be due to incompatibility with indigenous *S. erythraeus* plasmids. Conversely, stable transformation was not the result of plasmid or host mutations. Transformation instability or plasmid copy number thus prevented successful shotgun-cloning of DNA that complemented the *eryD* mutation, which blocks the biosynthesis of erythromycin A, because only plasmid DNA without an insert could be isolated from a $Thio^r$ Ery^+ clone.

The development of gene-cloning methods for the *Streptomyces* has depended heavily on the use of polyethyleneglycol (PEG)-induced transformation of *Streptomyces* protoplasts with plasmid or phage DNA. Satisfactory transformation of *Streptomyces erythraeus*, which produces the macrolide antibiotic, erythromycin A[1], could not be achieved using the protocols developed for *Streptomyces lividans*[2~4] and more recently for *Streptomyces ambofaciens* and *Streptomyces fradiae*[5]. Therefore, we studied several parameters which affected transformation of *S. erythraeus* and developed a method for transforming the UW278 (*met*-11, *eryD*-24) strain with an efficiency of *ca.* 4×10^6 transformants/μg of plasmid DNA. During our work we also observed a very high instability of the thiostrepton-resistance phenotype among transformants of this and other *S. erythraeus* strains. This phenomenon may be one reason we were unsuccessful in shotgun-cloning *ery* genes by complementation of the *eryD* mutation[6] since we could not recover plasmid DNA containing the complementing DNA from an $EryA^+$ transformant†.

Experimental

Bacterial Strains and General Reagents

The wild type *S. erythraeus* NRRL 2338 (UW22), 2359, 2360 and 2361 strains were obtained from the culture collection of the Northern Regional Research Laboratory, Peoria, IL. The *S. erythraeus* UW278 Ery^- mutant strain was derived from the NRRL 2338 strain as described by WEBER *et al.*[6] *S. lividans* TK24[8] was obtained from DAVID HOPWOOD, John Innes Institute, Norwich, England.

Biochemical reagents and enzymes were purchased from Sigma Chemical Co., St. Louis, MO; Boehringer-Mannheim, Indianapolis, IN; Bethesda Research Laboratories, Gaithersberg, MD; or New England Biolabs, Beverly, MA. PEG was purchased from J. T. Baker, Inc., Phillipsburgh, NJ; Koch-Light, Ltd., Haverhill, Suffolk, UK; Fluka Chemical Co., Hauppauge, NY; or Sigma. Thiostrepton was obtained from E. R. Squibb and Co., Princeton, NJ, and erythromycin A from Abbott Laboratories, N. Chicago, IL.

Growth Media and Conditions

All *Streptomyces* bacterial cultures were grown as described by CHATER *et al.*[4] or WEBER *et al.*[6]

† A preliminary account of this work has been presented elsewhere[7].

using one of the following media. Media constituents were purchased from Difco Laboratories, Detroit, MI, unless a different source is specified. SGGP*: Bacto peptone 4 g, yeast extract 4 g, Casamino acids 4 g, glycine 2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, H_2O to 960 ml, with pH adjusted to 7.0~7.2. After sterilization, 20 ml of a 50% solution of glucose and 20 ml of a 0.5 M solution of KH_2PO_4 were added. (SGGP was SGGP* without the Casamino acids and an equally satisfactory growth medium for *S. erythraeus*.) SLM-3: soluble corn starch (Mallinckrodt, Paris, KY) 10 g, corn steep liquor 5 g, yeast extract 3 g, $CaCO_3$ 3 g, $FeSO_4 \cdot 7H_2O$ 12 mg, agar 20 g, H_2O to 1 liter, with pH adjusted to 5.8 using solid Na_2CO_3. R2T20 was R2T[9] with 20% sucrose.

Protoplast Transformation Procedure

The Optimized Procedure is as Follows: A baffled 250-ml Erlenmeyer culture flask containing 50 ml of SGGP* was inoculated with 0.2 ml of mycelial cells from a seed culture previously grown in SGGP* to stationary phase and frozen at −80°C. This culture was grown at 30°C to an OD_{600} of 0.6 to 0.9 (reading was taken after a 10× dilution of the cells) and the cells were recovered by centrifugation in a Sorvall SS34 rotor at 13,000 rpm for 10 minutes. The pellet was washed once with a 10.3% sucrose solution and the cells were suspended in 8 ml of modified P medium (sucrose 20 g, K_2SO_4 25 mg, 1 M $MgCl_2 \cdot 6H_2O$ 0.5 ml, trace elements[9] 0.2 ml, H_2O to 89 ml: After sterilization, the following solutions were added; 0.5% KH_2PO_4 1 ml, 1 M $CaCl_2 \cdot 2H_2O$ 20 μl, 0.25 M pH 8 TES buffer 10 ml) and transferred to two 13 ml plastic tubes. Eight ml of a solution of lysozyme (2 mg/ml) in modified P medium was added to the cells and the mixture was incubated at 30°C for *ca.* 15 minutes with occasional mixing within a 5 ml disposable pipet until microscopic analysis showed that protoplast formation was complete. After filtering the mixture through cotton wool[9], the protoplasts were sedimented in a 13 ml Falcon 2057 (Falcon, Oxnard, CA) tube by centrifugation in a Sorvall SS34 rotor at 3,800 rpm for 8 minutes and washed once by resuspension in 10 ml of the modified P medium and recentrifugation. The protoplasts were resuspended to 10^9 to 10^{10} protoplasts/ml in modified P medium containing 10% DMSO, and 0.1 to 0.2 ml aliquots in 4 ml Falcon 2054 tubes were slowly frozen at −80°C by inserting the tubes in an ice filled beaker before placing them in the low temperature freezer.

For transformation, 2 to 4×10^8 frozen protoplasts were quickly melted in a warm H_2O bath at <40°C and spun down in an Eppendorf microcentrifuge for 10 seconds. The supernatant was decanted and the pellet was resuspended in the remaining solution, then 10 to 100 ng of plasmid DNA in 10 μl of TE-10% sucrose[9] was added and mixed with the cells by gently tapping the tip of the Eppendorf tube. J. T. Baker PEG 3350 - T buffer[3,9] (1: 3), 100 μl, immediately was added and the mixture admixed by drawing it 3× into a 100 μl tip of a Gilson pipetman. The protoplast mixture or its serial dilution then was poured onto the surface of an R2T20 regeneration plate† which had been freshly made and dried in a laminar flow hood to 82~85% of its original weight, and the plate was overlayered with 3 ml of soft R2T20 medium (the agar in R2T20 medium was replaced with 0.7% of low gelling temperature agarose [Sigma type VII]) previously warmed to *ca.* 37°C. The regeneration plates were incubated upright at 28°C for 38~40 hours, then overlayered with 3 ml soft R2T20 containing enough thiostrepton to give a final concentration of 25 μg/ml in the plate. Thio^r transformants usually were scored after 2 to 4 days further growth at 28°C.

Plasmid DNA Isolation

Plasmid DNA was isolated on a small and large scale from *Streptomyces* sp. according to methods described by HOPWOOD *et al.*[9].

Shotgun-cloning in the EryD Mutant

Chromosomal DNA was isolated from the UW22 strain by an established method[9] and partially digested with *Mbo* I. This restriction digest was size-fractionated on a 10~40% sucrose gradient. The fractions containing 9~20 kb DNA fragments were combined and the nucleic acids were precipitated. Vector DNA was digested with *Bgl* II and treated with calf intestine alkaline phosphatase (Boehringer-Mannheim, molecular biology grade), then ligated with the insert DNA as directed[9]. After recovery of the DNA by precipitation, it was used to transform protoplasts of the UW278 strain as described

† The sucrose in this medium could be replaced with 0.5 M mannitol.

above (the R2T20 regeneration plates also contained 60 μg/ml of methionine). The Thio^r colonies were transferred to fresh R2T20 plates containing 25 μg/ml of thiostrepton and, after growth and sporulation, each colony was picked to a separate well of a 96-well microtiter plate filled with SLM-3 medium. After growth for 6 days at 28°C, each of the inoculated plugs was bioassayed for antibiotic production by transfer to L agar[9] (which is the same as Luria agar[10]) containing 5 μg/ml of thiostrepton and seeded with an erythromycin A sensitive, thiostrepton resistant *Bacillus subtilis* strain.

Results

Transformation System

Although we obtained erratic results when the transformation protocol developed for *S. lividans* by HOPWOOD and co-workers[2,3] was applied to the UW22 strain[7], this observation suggested that their protocol could be used as a starting point to develop a transformation system for various *S. erythraeus* strains. Moreover, during this work we found that pWOR109, a plasmid vector constructed from pJV1[11] by researchers at the Beecham Laboratories and the John Innes Institute[12], was the most suitable vector for use with *S. erythraeus*.

The initial experiments with *S. erythraeus* protoplasts were designed to seek a solution to their erratic regeneration and poor stability upon storage at −80°C. Regeneration efficiency was found to be good when protoplasts were made with the P medium of OKANISHI *et al.*[13] from mycelia at a late log to early stationary phase of growth and were regenerated on the R2T medium[6] having a 20% sucrose concentration, following the precedents of IKEDA *et al.*[14] and SHIRAHAMA *et al.*[15]. The resulting transformation efficiency was *ca.* 5×10^4 transformants per microgram of pWOR109 DNA as shown in Fig. 1. The empirical adjustment of the Ca^{2+} and Mg^{2+} ion concentrations gave a further improvement in the regeneration efficiency (Fig. 2), which resulted in the adoption of a modified P medium containing sucrose 20%, Ca^{2+} 0.2 mM and Mg^{2+} 5 mM for all further experimentation. Finally, the addition of 10% DMSO to this medium was found to increase the stability and transformability of protoplasts stored at −80°C (Fig. 3a), and to give *ca.* 4.5×10^6 transformants per microgram of pWOR109 DNA when the

Fig. 1. Effect of culture age on the regeneration and transformation with pWOR109 of *S. erythraeus* UW278 protoplasts.

Protoplast regeneration on P medium (■), protoplast transformation with pWOR109 (▲), OD_{600} of culture after correction for a 10× dilution (○).

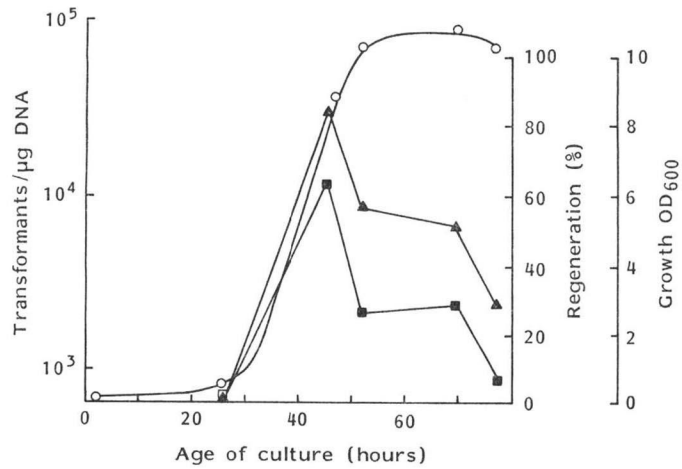
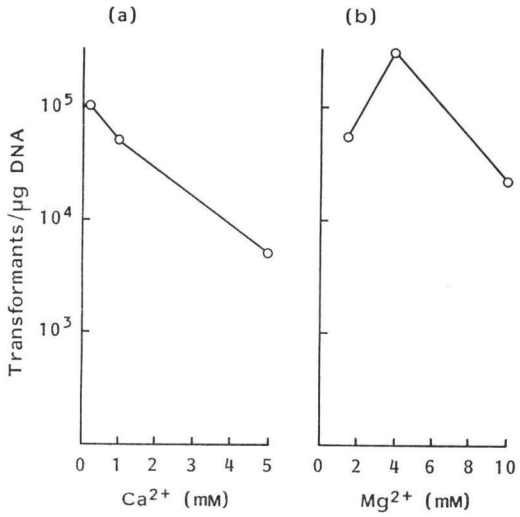


Fig. 2. Effect of divalent ion concentrations on transformation of *S. erythraeus* UW278 protoplasts with pWOR109.

(a) Variation of Ca^{2+} concentration at a constant 2 mM Mg^{2+} concentration. (b) Variation of Mg^{2+} concentration at a constant 0.2 mM Ca^{2+} concentration.



protoplasts were transformed at 30°C rather than at 4°C[5] (Fig. 3b).

We next examined several other parameters that have been noted to have a beneficial effect on the regeneration or transformation of *Streptomyces* protoplasts[5,9,15]. The following observations are noteworthy. In contrast to the situation for *S. ambofaciens* and *S. fradiae*[5], the addition of protamine sulfate (1 to 3 μg) to the plasmid DNA before adding it to the protoplasts inhibited the transformation efficiency from about 2 to 250-fold (Fig. 4a). The addition of calf thymus DNA increased the efficiency slightly at 0.5 μg but at 1 to 3 μg resulted in no significant difference from the base value (Fig. 4b). We did not examine the effect of adding the protamine sulfate and calf thymus DNA together[5]. The addition of heparin (2 to 20 μg) had no significant effect on the transformation efficiency (Fig. 4c). The use of 1% rather than 0.2% glycine in the SGGP* medium gave approx. a 3-fold increase in transformation efficiency according to the results of one experiment, but 0.5 M mannitol or 0.55 M disodium succinate[15] in the regeneration medium were not significantly better osmotic stabilizers than 20% sucrose with respect to the effect on transformation efficiency. We did not test the P3 and PWP buffers or R3 media of SHIRAHAMA *et al.*[15] for regeneration or transformation of *S. erythraeus* protoplasts. Using the modified P medium with a pH of 8, rather than 7.2, resulted in approx. a 10-fold increase in transformation efficiency based on the results of one experiment. Replacing the T buffer (10 mM Ca^{2+})[9] with an equal volume of modified P medium gave a *ca.* 100-fold decrease in transformation efficiency. With 400 ng to 2 μg of plasmid DNA, the optimum transformation efficiency per microgram of DNA was obtained using 2 to 4×10^8 protoplasts, but on a transformant per protoplast basis it was optimum with 9×10^7 protoplasts.

Vector Stability

The results (Table 1) of transforming the UW22 or UW278 strains with seven different plasmid vectors, which were obtained before the transformation system had been fully optimized, show that the initial transformation efficiency was low for all vectors except pWOR109. This value increased greatly for pIJ922 and pHJL210 (data not shown), but only *ca.* 10-fold for pWOR109, when the plasmid DNA isolated from a *S. erythraeus* transformant was used to retransform the same or a different strain. This indicates that DNA restriction and modification has a major influence on the transformation efficiency. In contrast, the stability of the plasmids in the primary transformants does not seem to be affected by restriction-modification since the stability of transformants did not increase when the transforming DNA was isolated from *S. erythraeus*.

We determined the stability of the resulting $Thio^r$ phenotype by serial transfer of a random assort-

Fig. 3. Effect of DMSO concentration in the modified P medium on the transformation of *S. erythraeus* UW278 protoplasts with pWOR109.

(a) Variation in transformation as a function of storage time at −80°C with 0 (○), 5 (●), 10 (△) and 20 (▽) percent DMSO.

(b) Variation in transformation at 0°C (■) and 30°C (□) as a function of percentage of DMSO.

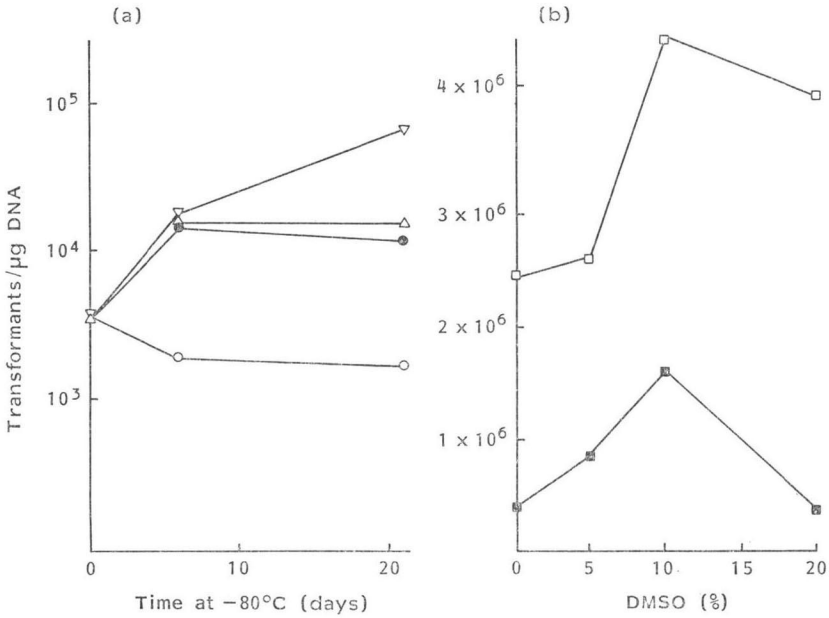
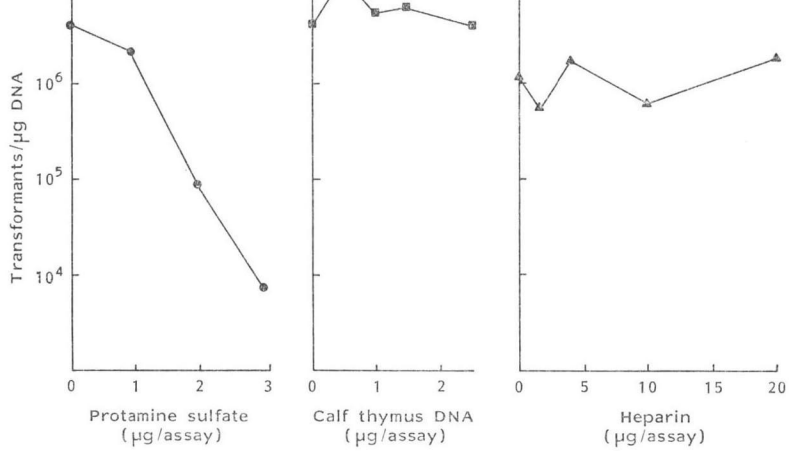


Fig. 4. Effect of the addition of protamine sulfate (●), calf thymus DNA (■) and heparin (▲) to the modified P medium on the transformation of *S. erythraeus* UW278 protoplasts with pWOR109.



ment of primary transformants from each experiment on R2T media with and without 25 μg/ml of thiostrepton. Testing 100 transformants revealed in most cases that the stability was very low since the majority of cells in a patch of growth failed to grow when transferred to the medium containing thiostrepton, but grew normally on the non-selective media. This was particularly true of pIJ702 trans-

Table 1. Transformation of *S. erythraeus* with plasmid vectors.

Plasmid	Host strain	Transformation efficiency per μg of DNA	Stability[a]
pIJ61[9),b]	UW278	6.0×10^2	−
pIJ702[9),b]	UW22	ND[c]	−
pIJ922[9),b]	UW278	6.0×10	−[d]
pIJ922[e]	UW278	2.3×10^4	−
pWOR109[b]	UW278	3.0×10^3	+
pWOR109[e]	UW278	3.8×10^4	+
pFJ105[28),b]	UW278	1.0×10^2	−
pHJL197[29),f]	UW278	0	
pHJL210[30),f]	UW278	8.0×10^g	+

[a] A "+" indicates that the Thio^r phenotype was stable when transformants were grown selectively; a "−" indicates that this phenotype was unstable when transformants were grown selectively (see text).
[b] DNA was isolated from *S. lividans*.
[c] ND: Not determined since the none of the large number of initially Thio^r colonies grew when transferred to fresh growth medium containing 25 μg/ml of thiostrepton.
[d] One stable Thio^r transformant was isolated.
[e] DNA was isolated from *S. erythraeus*.
[f] DNA was isolated from *Escherichia coli*.
[g] A much higher transformation efficiency was obtained when DNA isolated from *S. erythraeus* was used.

formants, all of which failed to grow if only the spores from primary transformants were transferred. An example of these observations is shown in Fig. 5 for UW22(pIJ61) and UW22(pWOR109) transformants. One of the pWOR109 transformants grew normally and exhibited a stable Thio^r phenotype when serially transferred selectively or non-selectively. In this case, a host mutation was not the reason for the stability of pWOR109 since the stability (or transformability) did not increase when the UW22(pWOR109) strain cured of its plasmid by protoplast regeneration was used as the host in a retransformation experiment.

Since the instability of the Thio^r phenotype suggests that the plasmids were not replicating properly or segregating uniformly, we screened representative transformants for the presence of the vector DNA by the method of KIESER[16]. Plasmid DNA could be isolated in all cases, except for pIJ702, but in amounts much less than expected from the reported plasmid copy numbers. The expected amount of plasmid DNA could be isolated only when the transformed cells had a very stable Thio^r phenotype, *i.e.*, when resistance to thiostrepton was maintained even when the transformed cells were subcultured non-selectively. From a stable UW22(pIJ303) transformant, both the vector and another plasmid having a similar intensity in ethidium bromide stained gels as pIJ303 were isolated. The new plasmid subsequently was found to exist in the UW22 strain and to have an approximate size of 20 kb, which suggests that it may be similar to pSE211, an 18.5 kb plasmid isolated from *S. erythraeus* by KATZ and co-workers[17]. Comparison of the restriction fragment patterns, obtained by single and double digestions with nine different endonucleases, between pIJ303 and pWOR109 isolated from *S. lividans* and from stable *S. erythraeus* transformants established that the structures of these two plasmid vectors were unaltered upon transformation into *S. erythraeus*, but that the structure of pIJ922 was greatly changed (data not shown). The UW278 strain could be retransformed by this altered pIJ922 with good efficiency (Table 1) but none of the transformants were stable. Consequently, the stability of pIJ303, pIJ922 and pWOR109 in the UW22 or UW278 transformants is not due to a modified plasmid or host mutation.

Finally, we examined three other erythromycin A producing strains of *S. erythraeus* to determine how

their transformation efficiency and stability compared with the UW22 and UW278 strains. As shown in Table 2, three different plasmids transformed the NRRL 2359, NRRL 2360 and NRRL 2361 strains with less than a 10-fold difference in transformation efficiency, but again only pWOR109 gave a large percentage of stable transformants. A high proportion of these pWOR109 transformants lost their Thio^r characteristic when transferred non-selectively however (data not shown). The results shown in Table 2 were obtained after the transformation protocol had been fully optimized and therefore, represent optimum results for transformation of *S. erythraeus*. It is interesting to note that the NRRL 2359 strain contains a large plasmid (L. KATZ, personal communication; A. D. GRUND and C. R. HUTCHINSON, unpublished results) whose DNA shows no homology to the plasmid DNA isolated from other wild-type

Fig. 5. Stability of primary transformants of *S. erythraeus* UW22 (pWOR109) and UW22 (pIJ61).

Plate A shows (top) ten patches each of two independent pWOR109 transformants and (bottom) ten patches of a pIJ61 transformant after replica plating the primary transformants to R2T medium.

Plate B shows this set of colonies after replica plating to the same medium with 25 μg/ml of thiostrepton. Note the patchy appearance of the pWOR109 transformant colonies and the complete lack of growth of pIJ61 transformant colonies.

Plate C shows ten patches each of five more pWOR109 transformants from the same experiment after replica plating to R2T with 25 μg/ml of thiostrepton.

Plate D is the same set of colonies after replica plating to R2T medium. Note that the ten patches of the one very stable pWOR109 transformant grew equally well on both plates. The expected amount of pWOR109 could be isolated from this transformant, but all other transformants yielded much smaller amounts of vector DNA.

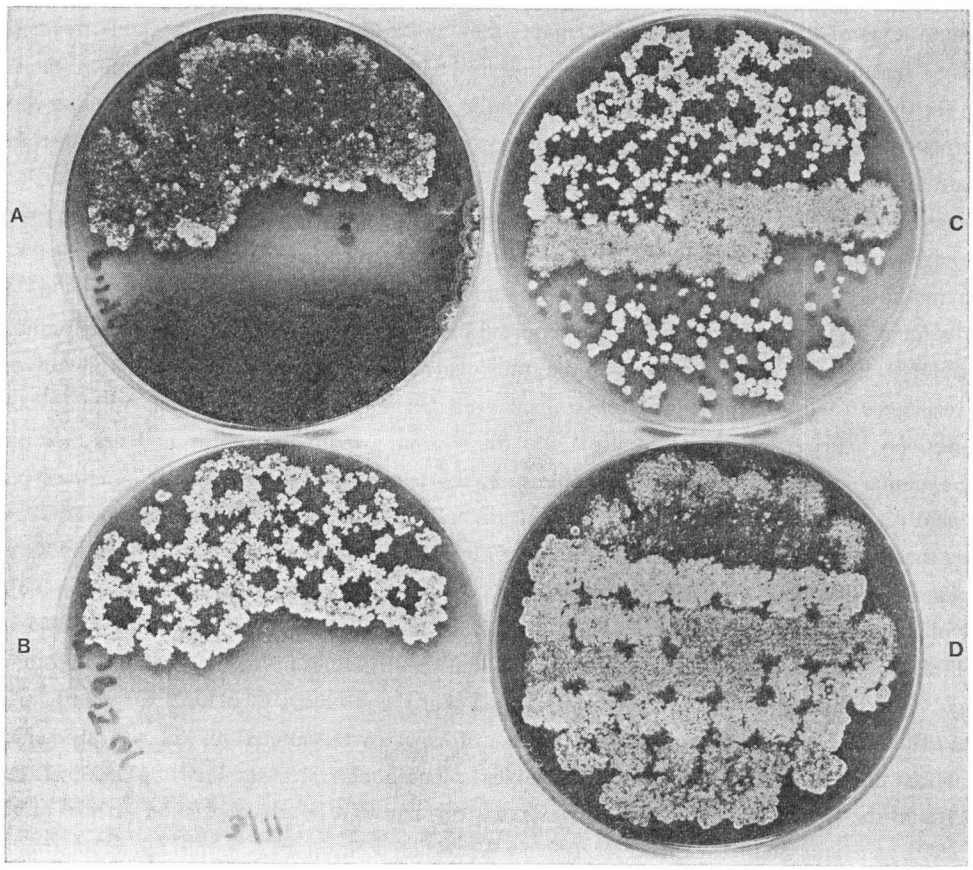


Table 2. Transformation of different *S. erythraeus* NRRL strains with plasmid DNA.

Plasmid[a]	Host strain NRRL	Transformation efficiency per μg of DNA	Stability (%)[b]
pWOR109	2359	1.2×10^4	70
	2360	1.2×10^3	95
	2361	1.2×10^3	90
pIJ922	2359	3.4×10^3	0
	2360	1.0×10^2	0
	2361	8.0×10^2	0
pFJ105	2359	1.0×10^4	0
	2360	1.0×10^3	0
	2361	3.0×10^3	0

[a] All DNA was isolated from *S. lividans*.

[b] The percentage of colonies which grew after two replica platings of the primary transformants on R2T20 medium with 25 μg/ml of thiostrepton (see text).

S. erythraeus strains like NRRL 2338 (L. KATZ, personal communication). Thus from the above results and the data in Table 2, which show that the NRRL 2359 strain was transformed slightly better than the other two strains, the moderate transformation efficiency and instability of transformants appears not to be due to incompatibility with indigenous *S. erythraeus* plasmids[17~19].

Shotgun-cloning of an *ery* Gene

We tested the suitability of pWOR109 for shotgun-cloning of *ery* genes by complementation of the *eryD* mutation in the UW278 strain (Experimental section). This mutation causes a blockade in the erythromycin A biosynthetic pathway between the formation of erythronolide B and erythromycin D and is believed to result in the disruption of deoxysugar biosynthesis[6]. One of the Thio^r clones, which also exhibited the Ery$^+$ phenotype, was shown by solvent extraction of culture medium and TLC analysis of the extracted materials to produce erythromycin A. Furthermore, the Thio^r and Ery$^+$ phenotypes were lost simultaneously when this clone was grown selectively or non-selectively. Yet we were not successful in isolating plasmid DNA from this transformant that contained an insert (gel electrophoresis data), or that retransformed the UW278 strain to the Ery$^+$ phenotype. The only plasmid DNA isolable from the original Thio^r Ery$^+$ clone, or from *S. lividans* TK24 that had been transformed with this DNA, was unchanged pWOR109. Since neither the Ery$^+$ nor the Thio^r Ery$^+$ phenotypes could be stabilized by serial transfer under selective conditions, it appears that the "cloned *eryD* gene(s)" could not integrate stably into the chromosomal DNA by single or double crossovers.

Discussion

Since an efficient transformation system for *S. erythraeus* is needed to study the molecular biology of erythromycin A production now that *ery* genes are available through the pioneering accomplishment of BALTZ and co-workers[20], the results we report here provide valuable information. The most important parameters affecting the transformation of *S. erythraeus* protoplasts are the Ca^{2+} and Mg^{2+} concentrations in the hypertonic protoplasting buffer, and the physical state of the protoplasts. An optimum Ca^{2+} concentration of 0.2 mM is much lower than that in the P (25 mM Ca^{2+})[2,5] or L (2.5 mM Ca^{2+})[3] media used in other studies. The beneficial effects of DMSO on protoplast viability and transformability parallels the use of DMSO in studies of recombination in *Streptomyces coelicolor* by protoplast fusion[21,22]. A stimulative effect of DMSO also has been noted in the transformation of *Escherichia coli*[23] and *Acetobacter* sp.[24].

The underlying reasons for vector instability in *S. erythraeus* strains are unknown. Our results appear to discount some of the more obvious causes such as plasmid incompatibility or severe DNA restriction. On the other hand, when stable transformants were found, this was not due to plasmid or host mutations. Vector copy number or gene dosage effects related to expression of the *tsr* or *ery* genes could be an important factor, particularly with high copy number vectors, since in another laboratory the use of low copy number vectors with apramycin resistance[25] as the selectable marker has given more satisfactory results in transformation of *S. erythraeus* (ref 20, R. H. BALTZ, personal communication).

Vector instability may limit the possibilities for studies of *ery* gene expression in a homologous DNA background if the outcome of our shotgun gene-cloning experiment is indicative of a general problem. We are aware that pWOR109 has been used successfully for gene-cloning in other *Streptomyces* sp.[12], but that a problem identical to the one we describe was encountered when another vector derived from pJV1 was used for subcloning *ery* DNA in *S. erythraeus* (L. KATZ, personal communication). Consequently, it may become necessary to develop gene cloning vectors from the indigenous *S. erythraeus* plasmids[17~19] or bacteriophages[26,27], or the analysis and exploitation of *ery* gene function will have to be done largely in a heterologous host.

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