Biochemical and Biophysical Research Communications 383 (2009) 42–47

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel transformation system using a bleomycin resistance marker with chemosensitizers for *Aspergillus oryzae*

Satoshi Suzuki [a,b], Sawaki Tada [b], Mari Fukuoka [b], Hiroko Taketani [b], Yoshiki Tsukakoshi [b], Mayumi Matsushita [b], Kosuke Oda [a], Ken-Ichi Kusumoto [b], Yutaka Kashiwagi [b], Masanori Sugiyama [a,*]

[a] Department of Molecular Microbiology and Biotechnology, Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan
[b] National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

ARTICLE INFO

Article history:
Received 19 March 2009
Available online 24 March 2009

Keywords:
Aspergillus oryzae
ATP-binding cassette (ABC) transporter
Bleomycin
Chlorpromazine
Drug resistance marker

ABSTRACT

Aspergillus oryzae is resistant to many kinds of antibiotics, which hampers their use to select transformants. In fact, the fungus is resistant to over 200 μg/ml of bleomycin (Bm). By enhancing the susceptibility of *A. oryzae* to Bm using Triton X-100 as a detergent and an ATP-binding cassette (ABC) pump inhibitor, chlorpromazine, to the growing medium, we established a novel transformation system by Bm selection for *A. oryzae*. In a medium containing these reagents, *A. oryzae* showed little growth even in the presence of 30 μg Bm/ml. Based on these findings, we constructed a Bm-resistance expression cassette (*BmR*), in which *blmB* encoding Bm *N*-acetyltransferase from Bm-producing *Streptomyces verticillus* was expressed under the control of a fungal promoter. We obtained a gene knockout mutant efficiently by Bm selection, i.e., the chromosomal *ligD* coding region was successfully replaced by *BmR* using *ligD* disruption cassette consisted of *ligD* flanking sequence and *BmR* through homologous recombination.

© 2009 Elsevier Inc. All rights reserved.

Introduction

Many studies have been conducted on the use of genetic transformation systems in *Aspergillus oryzae*, and the introduction of additional host-vector systems is expected to lead to the elucidation of the entire molecular basis of the whole organism.

Filamentous fungi are generally resistant to a broad range of antibiotics and fungicides. Compared to other *Aspergillus* species such as *A. nidulans*, *A. niger*, and *A. flavus*, *A. oryzae* is resistant to more kinds of drugs. Thus, it is difficult to obtain transformants by selection with commonly used drugs, such as hygromycin B and G418 [1]. Only two drug resistance markers, the pyrithiamine resistance gene *ptrA* [2] and the carboxin resistance gene *AosdhB* (*cxr*) [3], have been successfully used in the transformation of *A. oryzae*. Another category of selection system, auxotroph complementation, has been used in the selection for genetic transformation [4]. Although such selection markers can be effective, each marker has its own advantages and disadvantages. It is therefore important to develop more selection markers for *A. oryzae* research.

To make drug resistance markers available for the isolation of *A. oryzae* transformants, it is essential to enhance the susceptibility of *A. oryzae* to known antifungal drugs, so that the general transformation methods established for other fungal species can be applied to *A. oryzae*.

Bleomycin (Bm) and phleomycin have been widely used for transformant selection in plant and animal cells as well as in fungi [5–7]. The Bm/phleomycin family of antibiotics is produced by *Streptomyces verticillus* and is known to cleave DNA and RNA strands of eukaryotes and prokaryotes, resulting in cell death. Resistance to the Bm/phleomycin family can be conferred by expressing several kinds of Bm-resistance genes, such as Tn5 *ble*, Sh *ble*, *blmA* [8] and *blmB*. *blmB* encodes BAT, a Bm *N*-acetyltransferase from *S. verticillus* [9]. NIH3T3 cells transformed with a mammalian vector carrying *blmB* exhibit resistance to Bm [10]. Although 200 μg Bm/ml or 100 μg phleomycin/ml inhibits the growth of *A. oryzae*, these antibiotics have not yet been used for genetic transformation in this species, since the resistance levels were about 2–10 times higher than those in other filamentous fungi in which these drugs were successfully used [5–7].

In this report, we demonstrate that agents modulating membrane activity, such as detergent and pump inhibitors, sensitize *A. oryzae* to a lower concentration of Bm. Then, we describe a transformation system for *A. oryzae* using *blmB* as a selection marker for Bm resistance.

Materials and methods

Microorganisms and media. *Aspergillus oryzae* strains: RIB40, niaD300 (provided by the National Research Institute of Brewing,

* Corresponding author. Fax: +81 82 257 5284.
E-mail address: sugi@hiroshima-u.ac.jp (M. Sugiyama).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.03.108

Japan). Media: low-concentration malt extract polypeptone (LMP) medium [1% malt extract and 0.05% polypeptone, if necessary, 0.007% Triton X-100, 0.1 mM chlorpromazine (Sigma–Aldrich Japan, Tokyo, Japan) and the specified concentration of bleomycin (Bm) sulfate (Cosmo Bio, Tokyo, Japan) were added], YPD medium (0.5% yeast extract, 1% polypeptone, 2% glucose), Czapek Dox (CD) medium (0.6% $NaNO_3$, 0.1% KH_2PO_4, 0.05% KCl, 2 mM $MgSO_4$, 1% glucose, and a 0.1% trace element solution consisting of 0.1% $FeSO_4{\cdot}7H_2O$, 0.88% $ZnSO_4{\cdot}7H_2O$, 0.04% $CuSO_4{\cdot}5H_2O$, 0.01% $Na_2B_4O_7{\cdot}10H_2O$, and 0.005% $(NH_4)_6Mo7O24{\cdot}4H_2O$). Bm is cytotoxic and must be treated carefully.

Cross-paper-strip assay. Experiments using paper strips placed crosswise were performed as described previously [11]. In the assay for synergistic activity of several agents with Bm, the assay plates containing conidia were prepared as follows: conidial suspensions of RIB40 (5×10^6 conidia/plate) were mixed with 10 ml aliquots of molten top agar (0.4% low-melting agarose) with LMP medium at 35 °C and immediately poured into Petri dishes to make agar plates (diameter 9 cm). A strip of filter paper (0.7 × 8 cm) containing 100 μg Bm and each one of the filter papers containing 10 μl Triton X-100, Tween 20, Tween 80, 10 mg saponin, and 20 mg verapamil (Sigma), diltiazem (Sigma), trifluoperazine (Sigma), or chlorpromazine (Sigma) were placed crosswise in the test agar plates. The plates were incubated for 3 days at 30 °C. The interaction between Bm and other agents at the crossing of the paper strips was assessed by observing the resulting inhibition area [12].

Transformation of A. oryzae. The transformation of *A. oryzae* was carried out according to a method described previously [13] with some modifications. Mycelia of *A. oryzae* RIB40 cultured overnight in YPD medium were used to generate protoplasts. The protoplasts were incubated with 20 μg DNA for 30 min. The transformed protoplasts were suspended in molten top agar (0.4% low-melting agarose, 0.8 M NaCl) with potato dextrose broth and poured onto same agar medium with 0.8 M NaCl. The protoplasts transformed with DNA were incubated for 2.5 or 3 days on nonselective regeneration agar medium to allow sporulation in the absence of Bm. The resulting conidia were collected and spread at approximately 1.2×10^7 conidia/plate on LMP–agar medium supplemented with 30 μg Bm/ml, 0.007% Triton X-100, and 0.1 mM chlorpromazine or LMP–agar medium supplemented with 200 μg Bm/ml. We refer to this method as conidial selection (CS). To compare the number of colonies obtained by CS and conventional protoplast selection, a protoplast suspension of strain niaD300 transformed with *niaD* vector was separated into two portions of equal volume. One portion was selected by conventional protoplast selection and the other was subjected to CS. The colonies obtained by the two selection methods were counted and the numbers were plotted on a scatter graph. The linear approximation was calculated by Microsoft Excel (Microsoft, Tokyo, Japan).

ligD disruption. Prior to construction of the *ligD* disruption cassette, we modified the base sequence of Bm-resistance gene, *blmB*, to reduce the G + C content (76–55%) for efficient amplification by PCR (Accession No. of the modified sequence of *blmB*: AB383158) without changing the amino acid sequence. A disruption cassette of *ligD* was constructed by fusion polymerase chain reaction (PCR) [14]. Three sections of the disruption cassette, namely, the 5′ flanking region of *ligD*, the modified *blmB* expression cassette (named *BmR*), and the 3′ flanking region of *ligD*, were amplified by PCR using three sets of primers: (ligD5F1) (5′-cggactttacttt gactcttttca-3′) and (BAT-ligD5RV) (5′-gagctagatatgttgtgggtaccg tcttattagaagcacgagtttcg-3′); (H2Bpro5Pst) (5′-ctgcagtcatttttgc gattgggaa-3′) and (sCtm3Kpn) (5′-ggtacccacaacatatctagctcaccg-3′); and (BAT-ligD3F) (5′-cccaatcgcaaaaatgactgcagacaggcaccttgcatcatcatc-3′) and (ligD3RV1) (5′-tcattgtgctgcgttgacagctc-3′). Then, the three fragments were mixed in a proportion of 1:3:1 and used as a template for fusion PCR using the primer set (ligD5F2) (5′-ata cccaatcttacatggccctac-3′) and (ligD3RV2) (5′-tcattgtgctgcgattga cagctc-3′). The fusion PCR product was then used in the transformation experiment. Bm-resistant transformants were selected using the CS method described above.

Statistical analysis of CS. If the number of Bm-resistant protoplasts is X and the total number of protoplasts is 4×10^7, then the probability (p) that a Bm-resistant conidium will be sampled is $X/(4 \times 10^7)$. When 1.2×10^7 (=n) conidia are spread on LMP–agar medium supplemented with Bm, the number of Bm-resistant conidia (d) on the medium is binomially distributed:

$$p(d|X) = Binom(d; n, p) = \binom{n}{d} p^d (1-p)^{n-d} \quad (1)$$

Then, the expectation of d is np. By substituting $p = X/(4 \times 10^7)$, we obtain the relation between X and d in (2):

$$X = 3.3 \times d \quad (2)$$

The expected number of independent types of transformants in d conidia was calculated using the following procedure.

First, consider the probability distribution $p(n_{indep} = x|d)$ for the number of independent types of transformants (n_{indep}) in the observed d conidia. If the number of Bm-resistant protoplasts is denoted by X, then the joint probability distribution is $p(n_{indep} = x|X, d)$. This joint probability distribution is calculated using the following algorithm:

(1) Draw d numbers from a set: {1, 2, 3, . . . ,X} with equal probabilities by random sampling with replacement.
(2) Count the number of unique elements n_{indep} in the d draws.
(3) Repeat (1) and (2) to obtain the distribution of n_{indep}.

Next, the marginal probability distribution $p(n_{indep} = x|d)$ is calculated as follows using the joint probability distribution $p(n_{indep} = x|X, d)$:

$$p(n_{indep} = x|d) = \sum X = 1^{\infty} p(n_{indep} = x|X, d)p(X, d)$$
$$= \sum X = 1^{\infty} p(n_{indep} = x|X, d)p(X|d)p(d) \quad (3)$$

Using Bayes' theorem [15], we obtain

$$P(X|d) = \frac{p(d|X)p(X)}{p(d)} \quad (4)$$

For $P(X)$, we assumed a uniform prior:

$$p(X) \propto 1 \quad (5)$$

By substituting Eqs. (1) and (5) into (4), and then Eq. (4) into Eq. (3), we obtain $p(n_{indep} = x|d)$.

Results and discussion

Selection medium to optimize susceptibility to Bm

To investigate the susceptibility of *A. oryzae* RIB40 to Bm, we first evaluated four media with a fixed Bm concentration of 200 μg/ml: (1) minimum medium Czapek Dox (CD) agar (pH 5.5); (2) CD agar (pH 7.5); (3) nutrient-rich medium YPD agar; and (4) LMP (data not shown). When medium 1, 2, or 3 was used, conidia of RIB40 germinated and grew continuously for 1 week. The effectiveness of Bm is reduced if the concentration of metal ions in minimal media is high. We therefore prepared a medium, designated LMP, composed of 1% malt extract and 0.05% polypeptone, derived from ATCC medium 324, which contains 2% malt extract and 0.5% peptone. Although the growth of RIB40 strain in LMP–agar medium without Bm was slower than that in YPD agar,

and the hyphal density of the colony was sparse, the colonies sporulated normally. In LMP–agar medium, 200 μg Bm/ml completely inhibited the growth of RIB40 strain.

Enhancement of susceptibility to Bm

Using the newly developed LMP medium, we attempted to increase Bm susceptibility by enhancing the influx of Bm but decreasing the efflux. A yeast mutant lacking Bm influx transporter exhibited high resistance to Bm [16]. Because we could not find any previous report on a Bm transporter in *A. oryzae*, we simply used detergent hoping to increase membrane permeability, though nonspecifically. We examined the toxicity of Bm in the presence of Triton X-100, Tween 20, Tween 80, and saponin, which were absorbed into paper strips and placed crosswise on plates. Fig. 1A shows that Triton X-100 displayed the strongest synergistic activity with Bm, while Tween 20 as well as Tween 80 displayed weak synergy and saponin did not display any synergy with Bm.

Next, we studied calcium channel blockers and calmodulin inhibitors, both of which have been shown to inhibit active drug efflux [17]. The calcium channel blocker verapamil is a common inhibitor of active drug efflux in multidrug-resistant cells. The calmodulin antagonist chlorpromazine is known to increase the susceptibility of the filamentous fungus *Botrytis cinerea* to fungicide by inhibiting ABC transporter activity [11]. We evaluated the effect of calcium channel blockers (verapamil and diltiazem) and calmodulin inhibitors (chlorpromazine and trifluoperazine) on the Bm susceptibility of the RIB40 strain. Experiments using paper strips placed crosswise showed that both calcium channel blockers displayed weak synergistic activity but both calmodulin inhibitors displayed strong synergistic activity with Bm (Fig. 1B). Based on the results shown in Fig. 1A and B, we selected Triton X-100 and chlorpromazine for further experiments.

We determined the maximum concentrations of Triton X-100 and chlorpromazine at which the growth of RIB40 was not affected; these concentrations were found to be 0.007% and 0.1 mM, respectively.

We then determined the minimum concentration of Bm that suppressed the germination of RIB40 conidia in the presence of Triton X-100 and chlorpromazine. Fig. 1C shows that 30 μg Bm/ml completely inhibited the growth of conidia in the presence of 0.007% Triton X-100 and 0.1 mM chlorpromazine, whereas the same concentration of Bm without Triton X-100 and chlorpromazine did not suppress conidial germination and growth. These

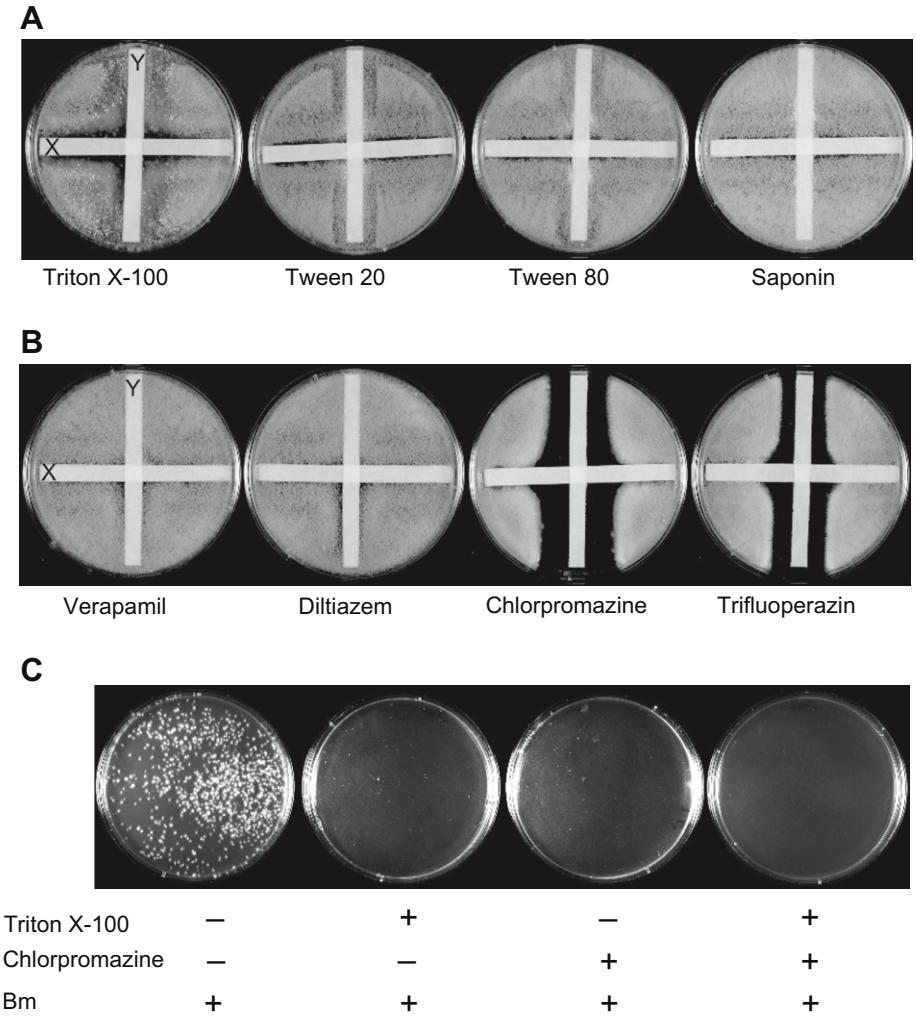

Fig. 1. Detergents and pump inhibitors synergistically increased the susceptibility of *A. oryzae* to bleomycin (Bm). (A) Synergistic activity between four kinds of detergents and Bm with respect to RIB40 conidia in experiments using crosswise-placed paper strips. The (Y) strips were impregnated with 10 μl of detergent and the (X) strips with 100 μg Bm. (B) Synergistic activity of four pump inhibitors with Bm with respect to RIB40 conidia in experiments using paper strips placed crosswise. The (Y) strips were impregnated with 20 mg of pump inhibitor and the (X) strips with 100 μg Bm. (C) Synergistic activity between 0.007% Triton X-100, 0.1 mM chlorpromazine, and 30 μg Bm/ml with respect to RIB40 conidia.

results suggest that *A. oryzae* may become sensitive to Bm/phleomycin at a low concentration by decreasing the efflux and increasing the influx of Bm.

Generation of ΔligD disruptant by homologous recombination with selection for Bm resistance

To confirm whether a gene disruptant could be obtained through transformation by Bm selection, we constructed a Δ*ligD* disruptant of *A. oryzae* by homologous recombination. LigD is one of the components of the repair pathway of DNA double-strand breaks in *A. oryzae* [18]. For selecting Δ*ligD* disruptants, we used modified *blmB*, encoding Bm *N*-acetyltransferase (BAT) derived from Bm-producing *S. verticillus*, as a Bm-resistant marker gene.

We constructed a BAT expression cassette (*BmR*) containing modified *blmB* under the control of the *A. oryzae* histone H2B promoter and the *A. nidulans sC* terminator (Fig. 2A). A *ligD* disruption cassette DNA fragment was also constructed by inserting *BmR* into the flanking region of *ligD*. The *ligD* was disrupted by *BmR* (Fig. 2B). Transformation was carried out by the CS method in the selection agar medium optimized in the experiment described in Fig. 1. We selected 72 Bm-resistant transformants, obtained from six independent experiments, from which 33 Δ*ligD* disruptants (46%) were identified by colony PCR and Southern blot analysis. This value is slightly larger than that (38%) reported previously by using the *argB* marker gene [19]. Southern blot analysis detected the expected bands at 7.5 kb in the genomic DNA of Δ*ligD* disruptant and 3.5 kb in the host strain, RIB40 (Fig. 2C). In selection agar medium containing 200 μg Bm/ml without addition of Triton X-100 and chlorpromazine, we could not obtain any Bm-resistant transformants from the same lot of transformed conidia as that described above (Fig. 2D). These results show that modified *blmB*

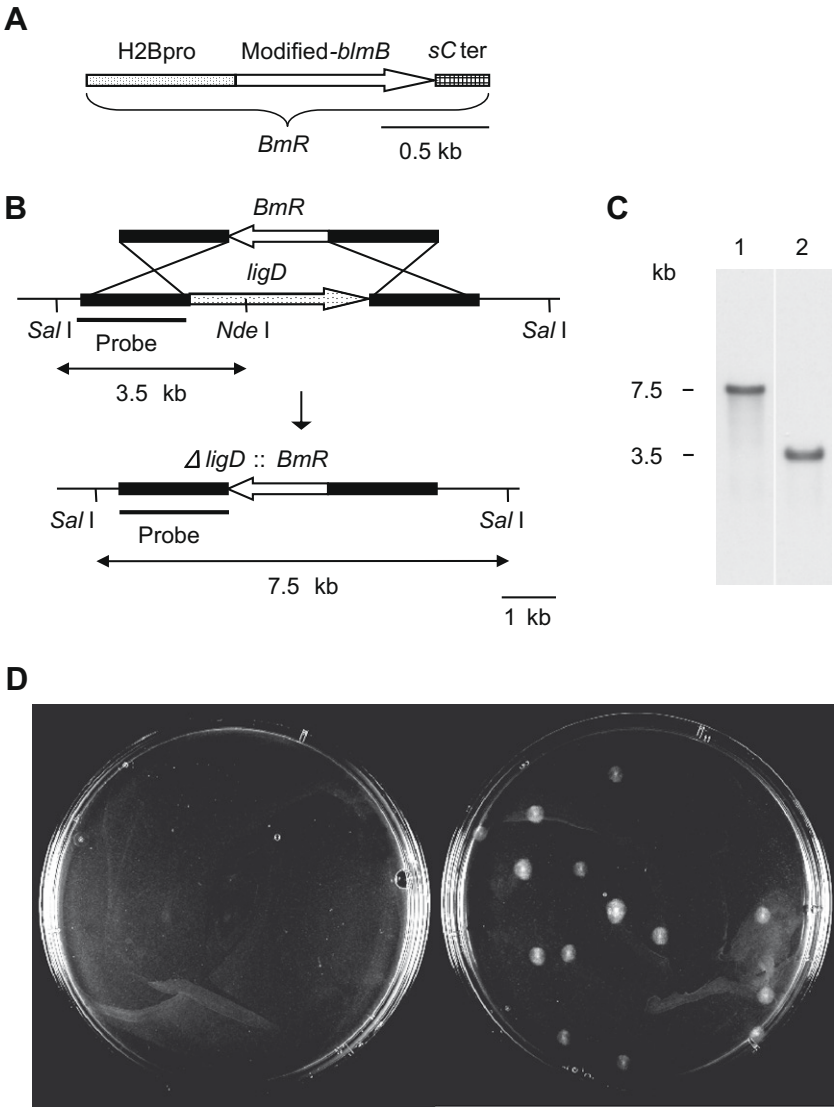

Fig. 2. The *blmB* expression cassette (*BmR*) inserted in the flanking sequence of the *ligD* and Bm-resistant transformants created by homologous recombination with the *A. oryzae ligD* locus. (A) Constitution of the modified *blmB* expression cassette (*BmR*). *Abbreviations:* the histone H2B promoter of *A. oryzae* (H2Bpro), the terminator of the *A. nidulans sC* gene (*sC*ter). (B) Strategy for homologous recombination of *A. oryzae* for *ligD* gene replacement by *BmR*. (Top) *BmR* (open arrow) inserted in *ligD* flanking sequence (solid bar). (Middle) *ligD* (dotted arrow) and flanking sequence (solid bar) in the host chromosome. (Bottom) *BmR* inserted into chromosomal *ligD* by homologous recombination. The horizontal bar indicates the hybridization positions of the probe to confirm gene replacement by Southern blot analysis. (C) Southern blot analysis of genomic DNA from the transformant and host strain. Each lane contained SalI and NdeI-digested genomic DNA of the transformant (lane 1) and the host strain RIB40 (lane 2). Two kilobases of the 5′ flanking region of *ligD* as a probe is indicated in (B). (D) Bm-resistance selection of the transformant by conidial selection. A conidial suspension obtained in a single transformation experiment was separated into two equal volumes and spread on two kinds of agar medium. (Left) The LMP–agar medium containing 200 μg Bm/ml without Triton X-100 and chlorpromazine. (Right) The LMP–agar medium containing 30 μg Bm/ml with Triton X-100 and chlorpromazine.

can be used as a selection marker through integration into the host chromosome by homologous recombination. Moreover, a reason for the higher disruption efficiency may be that the sequence in *BmR* that is homologous to the *A. oryzae* genome (only 800 bp of histone H2B promoter) is shorter than the auxotrophic marker gene from *A. oryzae*.

In this study, we showed that the enhanced susceptibility of *A. oryzae* cells to antibiotics was the key factor in developing a novel transformation system using drug resistance markers in this highly drug-resistant microorganism. We successfully established a transformation system for *A. oryzae* by using selecting markers for Bm resistance with the aid of agents that modulate membrane activity. To our knowledge, this is the first report in which an antibiotic resistance gene of bacterial origin has been used as a selection marker for *A. oryzae* transformation. A high concentration of Bm/phleomycin has been known to suppress the growth of *A. oryzae* and a gene for resistance to these antibiotics is already available, and therefore, many efforts have been made previously to develop a transformation system in *A. oryzae* based on Bm resistance [1,6]. Although successful transformation of strain U1638 using a phleomycin resistance gene was reported [20], the U1638 strain was generated by exposing *A. flavus* ATCC44310 to ultraviolet irradiation [21] and was not the so-called *A. oryzae* strain derived from Japanese fermentation products, which is clearly different from *A. flavus*.

Selection using a drug resistance marker could be applied essentially to any host strain, even to strains that have not yet been developed for auxotrophic mutants. Even if all auxotrophic markers have already been used up by several gene integration or gene disruption experiments in the host strain carrying multiple auxotrophic mutations, a drug resistance marker could still be used as a tool to manipulate another gene. To overcome the marker depletion problem, the *pyrG* marker recycling method was recently reported [19]. *pyrG* marker recycling is effective and enables infinite cycles of gene manipulation, but in each cycle, additional selection with 5-fluoro-orotic acid is required to recover the *pyrG*$^{-}$ mutant, which takes 5–8 days. It will be possible to choose *pyrG* marker recycling or drug resistance markers depending on the intended use.

We selected Bm-resistant transformants by the CS method in this study, because we could not obtain any Bm-resistant transformants by conventional protoplast selection. Post-transformation incubation is necessary to express a sufficient amount of the resistance gene; however, nonselective incubation causes protoplast aggregation and decreases susceptibility to Bm. To overcome this, the CS method was developed in this study. The number of transformants obtained by CS, as estimated by statistical methods, was slightly smaller than the number obtained by conventional protoplast selection (Eq. (2)) and the experimental data on model transformation by the *niaD* marker agreed with the statistical estimation data (Fig. 3). However, the CS method was efficient enough to allow us to obtain an adequate number of transformants in each experiment. Although the CS method involves more complex steps, such as collection of conidia, than conventional protoplast selection, the total number of experimental days needed to obtain transformants was similar to, or in some cases, less than that needed for protoplast selection. For example, conidiation of the first nonselective culture takes 2.5–3 days, and Bm-resistant colonies appear after 2 days on selection agar medium, whereas in case of protoplast selection of the niaD marker, nitrate-assimilatable colonies appear after a week. Moreover, the expected number of independent transformants calculated by equations described in Materials and methods shows that the proportion of redundancy of the CS colony derived from an identical transformed protoplast was negligible. For example, if eight colonies are obtained by CS, two of eight would originate from an identical protoplast; thus, a total of seven independent transformants can be obtained. However, we also recognize a critical defect of the CS method when studying sporulation-deficient mutants. Our objective in future is to develop Bm selection by conventional protoplast selection.

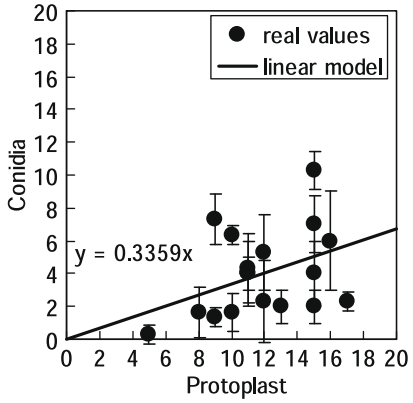

Fig. 3. Comparison of transformation efficiency by CS and conventional protoplast selection. The *X* axes: numbers of colonies obtained by conventional protoplast selection, the *Y* axes: numbers of colonies obtained by CS. Data for the *Y* axis are shown as mean ± standard deviation of three independent CS experiments.

We consider that the present detergent–pump inhibitor combination should work well with other drugs and in other organisms, and that the concept that it represents—the application of chemosensitization to genetic transformation systems—will contribute to the development of transformation systems in other drug-resistant organisms.

Acknowledgments

We wish to thank Dr. N. Tsuchida (Tokyo Medical and Dental University, Japan) for helpful comments to improve the manuscript and Dr. L. Clowney (NFRI) for improving the statistical analysis section of the manuscript. This work was supported in part by a Grant-in-Aid from the Ministry of Agriculture, Forestry, and Fisheries, Japan (Bio Design Project), Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

References

[1] K. Gomi, K. Kitamoto, C. Kumagai, Transformation of the industrial strain of *Aspergillus oryzae* with the homologous *amdS* gene as a dominant selectable marker, J. Ferment. Bioeng. 74 (1992) 389–391.
[2] T. Kubodera, N. Yamashita, A. Nishimura, Pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*: cloning, characterization and application as a dominant selectable marker for transformation, Biosci. Biotechnol. Biochem. 64 (2000) 1416–1421.
[3] Y. Shima, Y. Ito, S. Kaneko, H. Hatabayashi, Y. Watanabe, Y. Adachi, K. Yabe, Identification of three mutant loci conferring carboxin-resistance and development of a novel transformation system in *Aspergillus oryzae*, Fungal. Genet. Biol. 46 (2009) 67–76.
[4] F.H. Jin, J. Maruyama, P.R. Juvvadi, M. Arioka, K. Kitamoto, Development of a novel quadruple auxotrophic host transformation system by *argB* gene disruption using *adeA* gene and exploiting adenine auxotrophy in *Aspergillus oryzae*, FEMS Microbiol. Lett. 239 (2004) 79–85.
[5] B. Austin, R.M. Hall, B.M. Tyler, Optimized vectors and selection for transformation of *Neurospora crassa* and *Aspergillus nidulans* to bleomycin and phleomycin resistance, Gene 93 (1990) 157–162.
[6] P.J. Punt, C.A. van den Hondel, Transformation of filamentous fungi based on hygromycin-B and phleomycin resistance markers, Methods Enzymol. 216 (1992) 447–457.
[7] Z.M. He, M.S. Price, G.R. Obrian, D.R. Georgianna, G.A. Payne, Improved protocols for functional analysis in the pathogenic fungus *Aspergillus flavus*, BMC Microbiol. 7 (2007) 104.
[8] M. Sugiyama, C.J. Thompson, T. Kumagai, K. Suzuki, R. Deblaere, R. Villarroel, J. Davies, Characterization by molecular-cloning of 2 genes from *Streptomyces verticillus* encoding resistance to bleomycin, Gene 151 (1994) 11–16.
[9] M. Sugiyama, T. Kumagai, M. Shionoya, E. Kimura, J.E. Davies, Inactivation of bleomycin by an N-acetyltransferase in the bleomycin-producing strain *Streptomyces verticillus*, FEMS Microbiol. Lett. 121 (1994) 81–85.

[10] M. Sugiyama, M. Tanaka, T. Kumagai, A mammalian vector carrying the bleomycin N-acetyltransferase gene from bleomycin-producing *Streptomyces verticillus* as a selective marker, J. Antibiot. (Tokyo) 52 (1999) 895–899.
[11] K. Hayashi, H.J. Schoonbeek, M.A. De Waard, Modulators of membrane drug transporters potentiate the activity of the DMI fungicide oxpoconazole against *Botrytis cinerea*, Pest Manag. Sci. 59 (2003) 294–302.
[12] M.A. De Waard, J.G.M. Vannistelrooy, Antagonistic and synergistic activities of various chemicals on the toxicity of fenarimol to *Aspergillus nidulans*, Pestic. Sci. 13 (1982) 279–286.
[13] K. Gomi, Y. Iimura, S. Hara, Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene, Agric. Biol. Chem. 51 (1987) 2549–2555.
[14] H. Kuwayama, S. Obara, T. Morio, M. Katoh, H. Urushihara, Y. Tanaka, PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors, Nucleic Acids Res. 30 (2002) e2.
[15] T. Bayes, An essay toward solving a problem in the doctrine of chances, Philos. Trans. R. Soc. Lond. 53 (1764) 370–418.
[16] M. Aouida, N. Page, A. Leduc, M. Peter, D. Ramotar, A genome-wide screen in *Saccharomyces cerevisiae* reveals altered transport as a mechanism of resistance to the anticancer drug bleomycin, Cancer Res. 64 (2004) 1102–1109.
[17] T. Tsuruo, in: I.B. Roninson (Ed.), Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells, Plenum Publishing Co., New York, 1991, pp. 349–372.
[18] O. Mizutani, Y. Kudo, A. Saito, T. Matsuura, H. Inoue, K. Abe, K. Gomi, A defect of LigD (human Lig4 homolog) for nonhomologous end joining significantly improves efficiency of gene-targeting in *Aspergillus oryzae*, Fungal. Genet. Biol. 45 (2008) 878–889.
[19] J. Maruyama, K. Kitamoto, Multiple gene disruptions by marker recycling with highly efficient gene-targeting background (DeltaligD) in *Aspergillus oryzae*, Biotechnol. Lett. 30 (2008) 1811–1817.
[20] S. Cheevadhanarak, G. Saunders, D.V. Renno, T.W. Flegel, G. Holt, Transformation of *Aspergillus oryzae* with a dominant selectable marker, J. Biotechnol. 19 (1991) 117–122.
[21] A. Kalayanamitr, A. Bhumiratana, T.W. Flegel, T. Glinsukon, A. Shinmyo, Occurrence of toxicity among protease, amylase, and color mutants of a nontoxic soy-sauce koji mold, Appl. Environ. Microbiol. 53 (1987) 1980–1982.