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BIOCATALYSIS

Engineering of *Rhodococcus* cell catalysts for tolerance improvement by sigma factor mutation and active plasmid partition

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Abstract Tolerance to various stresses is a key phenotype for cell catalysts, which are used widely in bioproduction of diverse valuable chemicals. Using the *Rhodococcus ruber* TH strain, which exhibits high nitrile hydratase activity, as the target cell catalyst for acrylamide production, we established a method to improve cell tolerance by stably introducing global transcription perturbation. The σ^{70} gene (*sigA*) of *R. ruber* was cloned and randomly mutated. An *R. ruber* TH3/pNV-*sigA*^{M} library containing additional *sigA* mutants was constructed and used for survival selection. The TH3/M4N1-59 mutant was selected by acrylonitrile/acrylamide double stress and exhibited a 160 % extension of the half-life of nitrile hydratase upon exposure to 40 % acrylamide. A redesigned *parDE*^{M} gene was introduced to *Rhodococcus* to accomplish stable inheritance of plasmids. A two-batch acrylonitrile hydration reaction was performed using the engineered cells as a catalyst. Compared to TH3, the acrylamide productivity of TH3/M4N1-59DE^{M} catalysis increased by 27.8 and 37.5 % in the first and second bioreaction batches, respectively. These data suggest a novel method for increasing the bioconversion productivity of target chemicals through *sigA* mutation of the cell catalyst.

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Introduction

The bioproduction of bulk or fine chemicals is receiving increasing attention. At the core of such processes are usually biocatalysts. When the substrate and product molecules of a bioreaction are sufficiently small to allow transport through the cell membrane, cells, not enzymes, are usually used as a biocatalyst in either the free or immobilized form. The cell catalyst is different from regular cell factories that produce intra- or extracellular metabolites by fermentation. A cell catalyst is a cell coated with enzymes, which is collected from a fermentation broth and utilized in a separate bioreactor. Both cell catalysts and cell factories face a common problem. That is, organic chemicals or other harmful environmental factors may have toxic effects on microbial cells or intracellular enzymes, which in turn may reduce bioprocess efficiency [18]. It is generally believed that cell-tolerance phenotypes are highly complex and that multiple genes are involved simultaneously [2]. Recent studies have presented a global strategy for regulating the tolerance phenotypes of cell factories at the transcriptional level using RNA polymerase sigma factors or TATA box-binding protein (SPT15), and have reported its feasibility in both yeast [1] and bacteria [2].

It is interesting to ask whether a transcription regulation strategy could be applied to cell catalysts to enhance their tolerance phenotype, especially in actinobacteria. Actinobacteria are single-celled and multicellular Gram-positive bacteria with a high DNA GC content, and are broadly used as biocatalysts for bioproduction or biodegradation of

various chemicals. *Rhodococcus* is a single-celled Gram-positive bacteria, and has been used successfully for large-scale acrylamide production.

The hydration conversion of acrylonitrile to acrylamide, catalyzed by nitrile hydratase, is conducted using *Rhodococcus* sp. N774 [7, 19], *Rhodococcus rhodochrous* J-1 [15, 21], *Rhodococcus* sp. M8 [3], and *R. ruber* TH [13] cell catalysts. However, this method has a number of drawbacks, including byproduct inhibition, the low thermal stability of nitrile hydratase, and the low acrylamide-tolerance of cells; these inhibit the efficiency of acrylamide production [4, 14]. To avoid byproduct (acrylic acid) formation during biocatalysis, the *amiE*-negative mutant *R. ruber* TH3 was constructed; this successfully reduced byproduct accumulation [13]. Introduction of stable salt-bridges into nitrile hydratase to strengthen its thermal stability is discussed elsewhere [11].

Here, the tolerance phenotype of an *R. ruber* TH3 cell catalyst toward acrylamide stress was investigated. Its sigma factor gene was cloned, expressed, and mutated to construct an engineered strain containing the mutant *sigA*. Approaches to high-throughput selection of mutants were investigated and a method for maintaining stable inheritance of the plasmids responsible for the improved tolerance phenotype was developed. Consequently, an optimal recombinant with improved acrylamide tolerance was obtained and used as a cell catalyst for acrylamide bioproduction.

Materials and methods

Strains, plasmids, growth conditions, and DNA manipulations

The bacterial strains and plasmids used in this study are listed in Table 1. *R. ruber* TH3 is an *amiE*-negative mutant, constructed by knocking out the amidase gene (converting acrylamide to acrylic acid) from *Rhodococcus ruber* TH. *R. ruber* TH3 and its genetically modified mutants were grown at 28 C in medium containing 20 g glucose, 1 g yeast extract, 7 g tryptone, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, and 0.5 g$MgSO_4 \cdot 7H_2O$ per liter. *E. coli* Top10 and XL 10-Gold were used as hosts for genetic cloning and were routinely grown at 37 C in Luria–Bertani (LB) medium [17]. Antibiotics were supplemented as needed at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 13.5 μg/ml; and kanamycin, 30 μg/ml. Plasmid and genomic DNA isolation, agarose gel electrophoresis, restriction enzyme digestion, DNA ligation, and DNA transformation were performed using standard procedures [17] or following the specific instructions in the manufacturer's

Table 1 Bacterial strains and plasmids used in this study

Strais/plasmids	Relevant genotypes	References
Strain		
R. ruber TH	The wild *Rhodococcus rubber*	Ma et al. [13]
R ruber TH3	*amiE*$^-$mutant (*amiE*::pPHU281, Tc^r)	Ma et al. [13]
TH	R ruber TH3 (*amiE*$^-$)+pNV-*sigA*	This study
TH3/AN10-9	R ruber TH3 (*amiE*$^-$)+pNV-*sigA*$^{AN10-9}$, Km^r,Tc^r	This study
TH/AM8-16	R ruber TH3 (*amiE*$^-$)+pNV-*sigA*$^{AN8-16}$, Km^r,Tc^r	This study
TH3/M4N1-59	R ruber TH3 (*amiE*$^-$)+pNV-*sigA*$^{M4N1-59}$, Km^r,Tc^r	This study
TH3/M4N1-59*DE*^{M}	R ruber TH3 (*amiE*$^-$)+pNV-*sigA*$^{M4N1-59}$-*parDE*^{M}, Km^r,Tc^r	This study
E. Coli XL10-Gold	Tet^{r}Δ(*mcrA*)*183*Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* The [F' proAB*lacI*qZΔ*M*15 Tn*10* (Tetr) Amy Cam^r]	Stratagene
E. Coli TP10	recA1 endA1 gyrA96 thi hsdR17 supE44 Δ(*lac-proAB*)/F' [*traD3 proA*$^+$*B*$^+$*lacI*^{a}*lacZΔM15*]	Sambrook and Russell 2001
Plasmids		
pMD18T	AT cloning vector, Ap^r	Taraka
pNV18.l	*E.coli—Nocardia* Shuttle vector	Chiba et al. 2007
pNVA	pNVl 8 1 derivative carrying a 1 4-kb*R ruber sigA* gene, Km^r	This study
pNV-*sigA*$^{AN10-9}$	pNVl 8 1 derivative carrying a 1 4-kb mutant*sigA* gene, Km^r	This study
pNV-*sigA*$^{AN8-16}$	pNVl 8 1 derivative carrying a 1 4-kb mutant*sigA* gene, Km^r	This study
pNV-*sigA*$^{M4N1-59}$	pNVl 8 1 derivative carrying a 1 4-kb mutant*sigA* gene, Km^r	This study
pNV-*sigA*$^{M4N1-59}$-par*DE*^{M}	pNVl 8 1 derivative carrying a 1 4-kb mutant*sigA* gene, and 0.8 kb *parDE*^{M}, Km^r	This study

protocol. Restriction enzymes and Taq DNA polymerase were purchased from Taraka (Dalian, China). PCR purification kit and Biospin gel extraction kit were purchased from Stratagene (San Jose, CA, USA) and Bioer technology (Hangzhou, China), respectively. The GeneMorph®II EZClone Domain Mutagenesis Kit used for random mutation of *sigA* was purchased from Stratagene.

Cloning of *sigA* from *R. ruber* TH

The PCR primers PA1 (5′-CTCAAGCAGATCGGCAAG-3′) and PA2 (5′-CGCGTTCAGTCCAGGTAGT-3′) were designed based on the alignment of conserved regions of *sigA* genes encoding the σ^{70} transcriptional factors from *R. jostii* RHA1(YP_706753), *R. erythropolis* PR4 (YP_002766263), *R. erythropolis* SK121 (ZP_04385127), *R. opacus* B4 (YP_002784000), and *Mycobacterium smegmatis* str MC2 155 (YP_887090) for amplification of a partial *sigA* gene fragment. Thermal asymmetric interlaced PCR (TAIL-PCR) was performed to amplify the full-length *R. ruber sigA* gene and its upstream fragment using the three nested sequence-specific primers SP1 (5′-CGAGCAGATG GTTCTTGG-3′), SP2 (5′-TGGCGAGTGAGACGACGAGT-3′), and SP3 (5′-T GGAGAACTT GTAACCCTTG GTG-3′), based on the partial *sigA* gene fragment from *R. ruber*, together with four shorter arbitrary degenerate primers (AD1, AD2, AD3, and AD4), as described in [12]. The PCR conditions included an initial denaturing step for 10 min at 95 °C and 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, followed by 10 min at 72 °C. TAIL-PCR conditions were modified as described in [12]. The PCR products were then ligated into the pMD18T cloning vector (Table 1). The presence of the correct insert was verified by colony-PCR. σ^{70} gene sequencing was performed by the Sino Company. Sequence assembly and ORF analysis was performed using the DNAMAN and basic local alignment search tool (BLAST) software on the NCBI Web site [9].

Mutant *sigA* library construction

The full-length *sigA* gene and its native promoter region were amplified by PCR using the primers PAE (5′-TCAGAA TTCTCGTTACAATGGTGCACAG-3′, the *Eco*RI site is underlined) and PAH (5′-ACAAAGCTTCGCGTTC AGTCCAGGTAGT-3′, the *Hind*III site is underlined). PCR products were purified using a Qiagen PCR cleanup kit, digested overnight by *Eco*RI and *Hind*III and ligated overnight into the *E. coli–Rhodococcus* shuttle vector pNV18.1 (Table 1) at the same restriction sites, resulting in the recombinant plasmid pNVA. By electroporation, pNVA was transformed into competent *R. ruber* TH3 (*amiE*$^-$) cells and plated on minimal-agar plates containing 50 μg/ml kanamycin, resulting in the recombinant strain *R. ruber* TH3 (*amiE*$^-$)/pNV-sigA, hereafter abbreviated TH3A. Using PAURM (5′-CGATCGTTACAATGGTGCACA-3′) and PA2 as primers, fragment mutagenesis was performed using the GenemorphII EZClone Domain Random Mutagenesis kit (Stratagene) with various concentrations of initial template to obtain low (0–4.5 mutations/kb), medium (4.5–9 mutations/kb), and high (9–16 mutations/kb) mutation rates, as described in the product protocol. After purification with a Qiagen PCR cleanup kit, these PCR fragments were utilized as primers and an EZClone reaction was performed using pNVA as a template. Products were digested with *Dpn*I to remove the pNVA vector that contained the native *sigA* gene, then transformed into *E. coli* XL10-Gold super-competent cells. Recombinant cells were spread on LB agar plates containing 50 μg/ml kanamycin and scraped off to create a liquid library. The liquid library was then cultured and plasmids were extracted to create a plasmid library.

High-throughput phenotype selection for acrylamide tolerance improvement

The plasmid library was transformed into electrocompetent *R. ruber* TH3 (*amiE*$^-$) cells by electroporation and spread onto solid plates containing 50 μg/ml kanamycin and 0.1 % (v/v) acrylonitrile, 0.8 % acrylamide, or both 0.1 % (v/v) acrylonitrile and 0.4 % acrylamide. This was the first-round high-throughput screen for surviving mutants under growth-lethal conditions. Also using lethal conditions, second-round high-throughput screening was performed in 12 deep-well microplates using 99 single colonies. Two superior mutants were selected for the third culture, performed in flasks, to further confirm the improved cell-tolerance phenotype toward acrylamide stress.

All mutant strains were cultured in the presence of 50 μg/ml kanamycin and compared to TH3A, which harbored the native sigma factor with the native promoter in the pNV18.1 plasmid, as described above. All mutant sigma factors were retransformed into a fresh strain background before final analysis, to differentiate the effects of sigma factors from those of any chromosomal mutations.

Selected mutant sigma factors were sequenced in pNV18.1 using the universal primers M13–47 (CGCCA GGGTTTTCCCAGTCACGAC) and RV-M (GAGCGG ATAACAATTTCACACAGG). Sequences were aligned and compared using DNAMAN version 6.0.

Cell culture and harvest, nitrile hydratase activity assays, and acrylamide-tolerance evaluation

Cell culture, harvest, and nitrile hydratase activity assays were performed as described previously [13]. For acrylamide-tolerance evaluation, mutant cells cultured for 48 h were collected by centrifugation at 12,000 rev/min for

10 min at 4 C, and washed twice with 50 mmol/l PBS (3 × PBS buffer; 8.0 g/l NaCl, 0.2 g/L KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4; pH 7.0), then resuspended in 3 g/l PBS buffer and soaked in 40 % acrylamide with shaking. At the appropriate times, 1-ml aliquots were removed, washed twice with 50 mmol/l PBS, and their nitrile hydratase activity was determined using GC, as described previously [13]. The starting nitrile hydratase activity before addition of acrylamide was defined as 100 %.

parDE^{M} introduction for stable plasmid inheritance in *Rhodococcus*

An 808-bp fragment named *parDE*^{M}, with an *Eco*RI restriction site at each end and containing the ORF of *ParDE* [16] responsible for stable plasmid inheritance from the large plasmid PK2 of *Pseudomonas* sp. RP4, was optimized based on the codon preference of *Rhodococcus* and artificially synthesized by Shanghai Sangon (China). The redesigned *parDE*^{M} was digested and inserted into the *Eco*RI site of plasmid pNV-*sigA*$^{M4N1-59}$, resulting in the recombinant plasmid pNV-*sigA*$^{M4N1-59}$-*parDE*^{M}. After sequencing verification, this was transformed into *R. ruber* TH3 to construct the recombinant strain *R. ruber* TH3/pNV-*sigA*$^{M4N1-59}$-*parDE*^{M} (hereafter, TH3/M4N1-59DE^{M}).

Plasmid stability was determined according to a previously described method [6] with slight modifications. Strain TH3/M4N1–59, containing plasmid pNV-*sigA*$^{M4N1-59}$, was used as a blank control. Strain TH3/M4N1–59DE^{M} was grown for 48 h in 10 ml of medium containing 30 μg/ml kanamycin. Cultures were diluted 1:100 in antibiotic-free medium and cultured for a further 36 h. This process was repeated 12 times. Each culture was diluted appropriately and spread onto solid plates with or without 50 μg/ml kanamycin. Colonies were scored after incubation for 2 days, and the stability of plasmids with or without *parDE*^{M} was determined as the proportion of colonies that were kanamycin-resistant.

Two-batch acrylamide bioproduction from acrylonitrile hydration

Laboratory-scale acrylonitrile hydration was carried out in a reaction mixture containing cell catalysts with 185 U/ml nitrile hydratase in 600 ml of deionized water (pH 7.0) in a 1,000-ml flask with three necks. Agitation (265 rev/min, IKA RW20 digital) was set through the middle neck. Substrate acrylonitrile was added drop-by-drop through one of the side-necks at a rate decreasing from 7.5 ml/min to 0. Reaction temperature, measured by a thermometer through another side-neck, was controlled at 16–20 °C in an ice-water bath. A hollow-fiber membrane module was linked to the flask for cell recovery after the acrylamide concentration in the first reaction batch reached 30 %, and the recovered cells were used as catalysts in the second reaction batch. Aliquots (970 μl) were removed at appropriate times and acrylamide concentrations were determined by GC after removal of cells by centrifugation (10 min, 12,000 rev/min).

Results

Cloning and analysis of *sigA* from *R. ruber* TH

The σ^{70} gene (*sigA*) in *Rhodococcus* was cloned and used to generate mutants. A 900-bp partial fragment encoding the sigma factor was first amplified using primers designed based on conserved domains. Next, a 1,947-bp sequence including the full *sigA* gene and the upstream fragment were amplified by TAIL-PCR (Fig. 1a). Sequence analysis showed that the product contained a single ORF of 1,344 bp (JF699276), which encoded the SigA protein (447 amino acids, ~49 kDa). Figure 1b shows the nucleotide sequence of the putative *sigA* promoter and the SigA N-terminal amino-acid sequence. The SigA sequence showed a high level of homology to those of all known sigma factors.

The *R. ruber* TH SigA showed 81.8, 85.3, 83.4, 83.1, and 75.1 % similarity to *R. jostii* RHA1 (YP_706753), *R. erythropolis* PR4 (YP_002766263), *R. erythropolis* SK121 (ZP_04385134), *R. opacus* B4 (YP_002784000), and *Mycobacterium smegmatis* str MC2 155 (YP_887090), respectively. Conserved domain analysis of deduced amino-acid sequences revealed that the *R. ruber* SigA protein and those from other bacteria had the conserved regions found in most sigma factor 70 s (Fig. 1c).

Insertion of the amplified wild-type *sigA* and the randomly mutated *sigA* alleles into the shuttle plasmid pNV18.1 resulted in the generation of plasmid pNV-*sigA* (pNVA) and a mutant plasmid library (pNV-*sigA*^{M}). Wild-type and mutant plasmids were transformed into *R. ruber* TH3 (*amiE*$^{-}$; hereafter, TH3). The mutant cell library contained ~10^6 transformants with diverse phenotypes, through enhanced expression of mutant *sigA*^{M} genes.

High-throughput screening of the SigA mutant library

Because the biohydration substrate (acrylonitrile) and product (acrylamide) are toxic to cell catalysts, they were selected as the environmental stress to induce tolerance evolution of mutant cells. A two-stage (solid–liquid) three-step survival selection strategy was presented, which includes colony culture on solid media, liquid culture in 12-well plates, and liquid culture in 300-ml flasks. At each stage, a lethal concentration of acrylonitrile, acrylamide, or

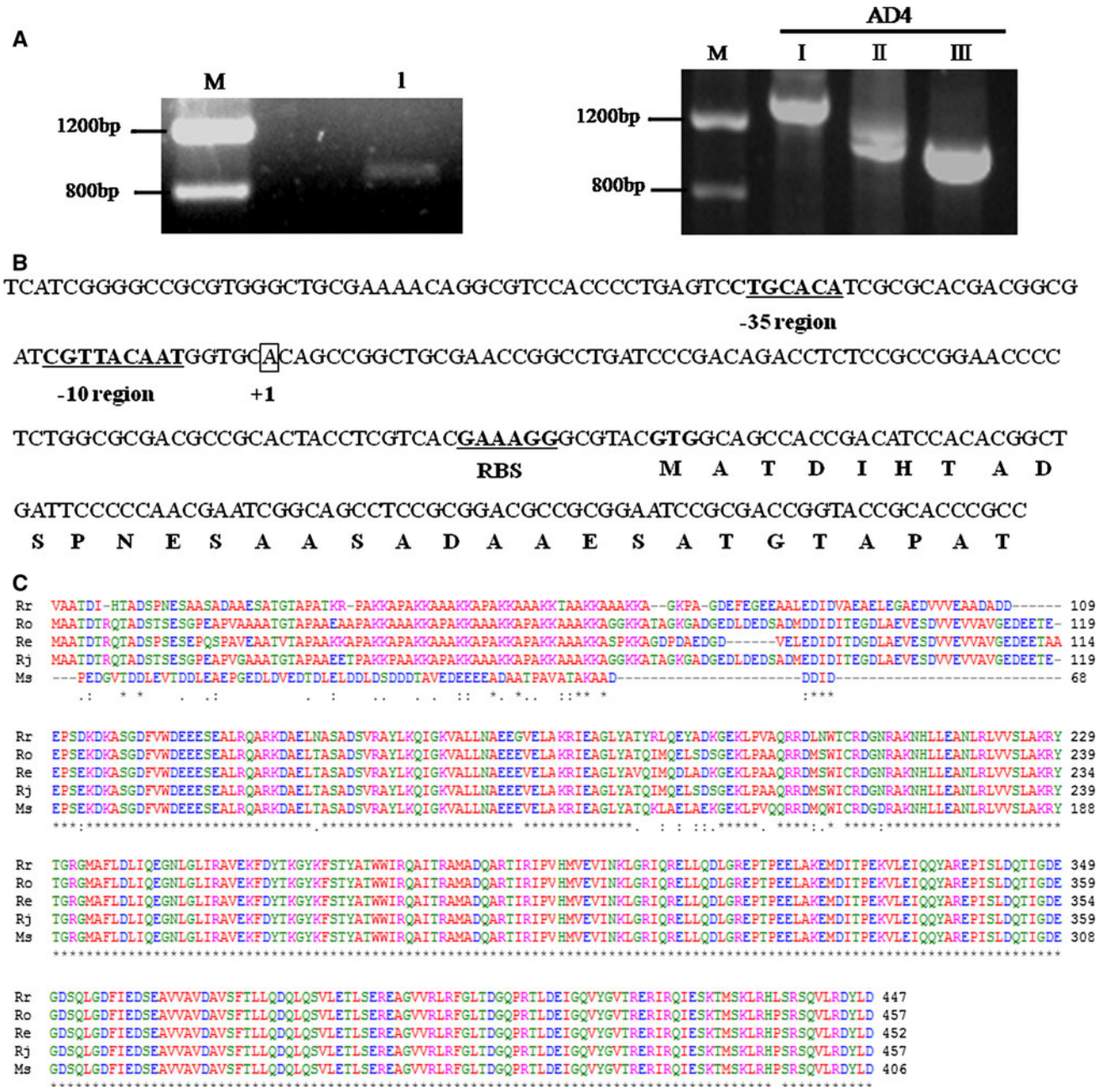


Fig. 1 Amplification products of *sigA* and an upstream fragment from *R. ruber* TH. Nucleotide sequence of the putative *sigA* promoter (**a**); N-terminal amino-acid sequences of the *sigA* gene product (**b**); and comparison of the deduced amino-acid sequence of the *R. ruber* SigA with sigma 70 factors of other bacteria (**c**). In **a**, M, DNA marker; lane 1, partial *sigA* fragment amplified by PCR with primers designed on the basis of conserved domains; lanes I, II, and III are the first, second, and third-round TAIL-PCR products amplified using the shorter arbitrary degenerate primer AD4 and the SP1, SP2, and SP3 nested sequence-specific primers, respectively. In **b**, the predicted -10 and -35 regions are underlined, the possible *sigA* transcription initiation site (+1) is boxed, and the putative ribosome binding site (RBS) is indicated in bold. In **c**, the boxed regions indicate areas conserved in most sigma factor 70 s. Rr, *R. ruber*; Ro, *R. opacus*; Rj, *R. jostii*; Re, *R. erythropolis*; Ms, *Mycobacterium smegmatis*

both, toward parent strain TH3 accumulated (Table 2). Use of 0.8 % acrylonitrile, 0.6 % acrylamide, or 0.1 % acrylonitrile, and 0.4 % acrylamide completely inhibited growth on solid media. In liquid culture, the lethal concentration of each stress was little changed.

The mutant SigA library cells were spread onto solid media containing lethal concentrations of acrylonitrile, acrylamide, or both. In total, 99 × 3 colonies were selected from each type of solid media and inoculated into liquid medium in 12-well plates, to which was added lethal

Table 2 Growth performance of TH3 supplemented with acrylonitrile, acrylamide, or both

	Acrylonitrile, %		Acrylamide, %		Acrylonitrile, acrylamide, %	
Colony culture on solid plate[a]	0	+	0	+	0, 0	+
	0.1	+	0.2	+	0.5, 0.2	±
	0.2	+	0.4	±	0.5, 0.4	±
	0.5	±	0.6	−	1.0, 0.2	±
	0.8	−	0.8	−	1.0, 0.4	−
	1.0	−	1.0	−	1.0, 0.6	−
Cell culture in liquid medium[b]	0	+	0	+	0, 0	+
	0.5	+	0.2	+	0.5, 0.2	+
	0.8	+	0.4	±	0.5, 0.4	+
	1.0	±	0.6	±	1.0, 0.2	±
	1.5	±	0.8	−	1.0, 0.4	±
	2.0	−	1.0	−	1.0, 0.6	–

Three plates were used at each level. [a] colonies cultured at 28 °C for 72 h. +, colony number was in the same order of magnitude as the control; ±, colony number was at least one order of magnitude lower and colonies were smaller; −, no growth. [b] cells cultured at 28 °C for 48 h in liquid medium. +, OD_{460} difference <10; ±, 10–20; −, >20

concentrations of acrylonitrile, acrylamide, or both. The growth of mutants under diverse conditions is summarized in Fig. 2. Strain TH3A, which exhibited enhanced native SigA expression, was used as a control.

Strains TH3/AN10-9 and TH3/AN10-53 (selected by acrylonitrile stress; Fig. 2a), AM8-16 and AM8-24 (selected by acrylamide stress; Fig. 2b), and TH3/M4N1-48 and TH3/M4N1-59 (selected by acrylonitrile/acrylamide double stress; Fig. 2c) all grew better than the control. We then further scaled-up cultures using higher acrylonitrile and acrylamide pressure, and monitored growth for 48 h (Fig. 3). Strains TH3/AN10-9, TH3/AM8-16, and TH3/M4N1-59 exhibited the greatest tolerance.

Mutant performance evaluation

Because acrylonitrile can be maintained at a very low level by feeding during acrylonitrile hydration, cell tolerance toward acrylamide is the major criterion for the evaluation of cell catalyst tolerance. Nitrile hydratase activity is another key performance metric. Using both TH3 and TH3A as controls, strains TH3/AN10-9, TH3/AM8-16, and TH3/M4N1-59 were simultaneously cultured in flasks for 48 h under stress-free conditions. Cells were harvested, subjected to nitrile hydratase activity measurement (Fig. 4a), and then observed for their deactivation behavior (Fig. 4b).

As shown in Fig. 4a, expression of native SigA in TH3A had no effect on nitrile hydratase activity compared to the TH3 control. In the TH3/AN10-9, TH3/AM8-16, and TH3/M4N1-59 mutants, nitrile hydratase activity increased by 10, 23, and 32 %, respectively. As shown in Fig. 4b, the nitrile hydratase deactivation rates of these mutants under 40 % acrylamide immersion were all slower than that of the TH3 and TH3A controls. The half-life of nitrile hydratase in TH3/AN10–9, TH3/AM8–16, and TH3/M4N1–59 extended 77, 130, and 160 %, respectively, with respect to TH3. As expected, strain TH3/M4N1–59, selected by acrylonitrile/acrylamide double-stress, was the best mutant. Recombinant plasmids were extracted from each of the three mutants and retransformed into a new TH3 host. The results were similar to those shown in Fig. 4, confirming that the improved cell-tolerance phenotype was conferred by the mutant sigma factor.

Mutations in the SigA protein were sequenced and analyzed (Fig. 4c). Five mutations, A59T, A96T, N140T, G164E, and T202I, were found consistently in each of the three mutant SigAs with a common trend of non-polar amino acid mutation to polar amino acids, with the exception of T202I. C-terminal truncations were also present in all three, due to the generation of a new stop codon by site-mutation of *sigA*. Such a mutant will alter the transcription of many genes, increase the expression of anti-stress genes, and enhance cell tolerance toward environmental stresses. Detailed analyses of the mutant sigma factor and transcriptome changes are in progress.

Enhancement of *Rhodococcus* plasmid stability by introduction of $parDE^M$

Owing to cost and environmental concerns, antibiotic use is not appropriate in large-scale fermentation of recombinant strains. Therefore, plasmid stability under antibiotic-free conditions is a key issue for any plasmid-containing recombinant strain, especially the non-familiar *Rhodococcus* host used here. To solve this problem, the $parDE^M$

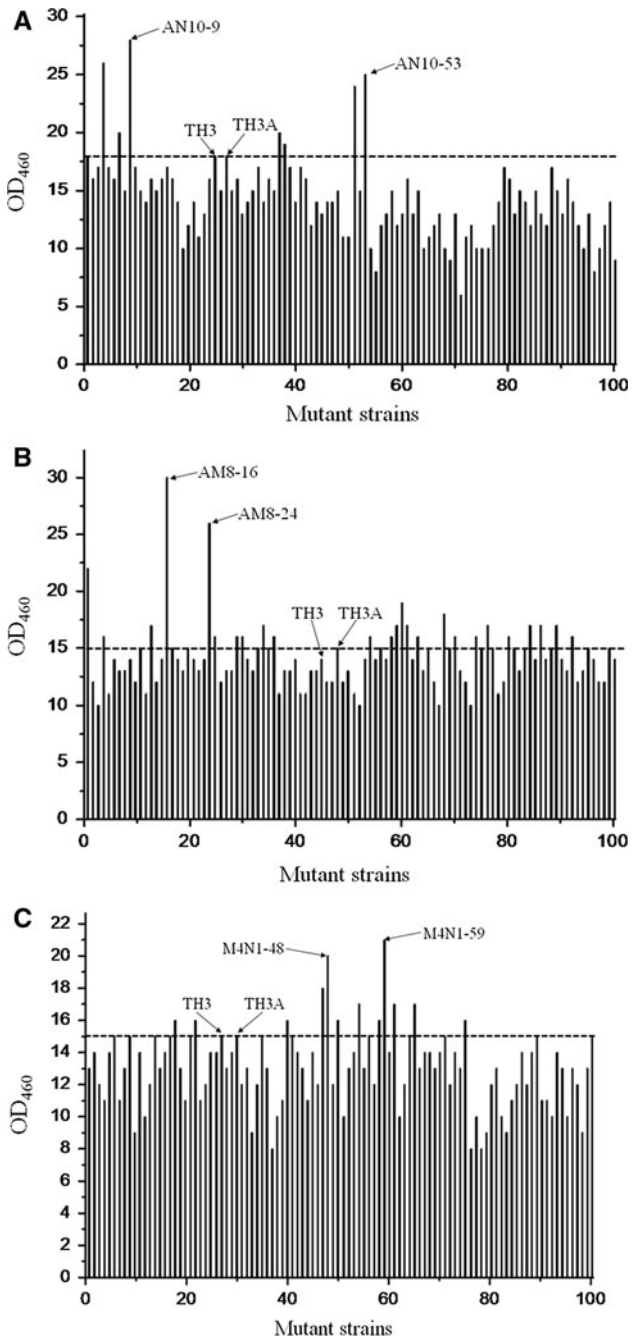


Fig. 2 High-throughput selection of mutants using growth phenotype under stress conditions. **a**, **b**, and **c** Mutant strains selected with 0.1 % acrylonitrile, 0.8 % acrylamide, or 0.1 % acrylonitrile and 0.4 % acrylamide, respectively

gene, responsible for active partitioning of plasmids from parent into daughter cells, was synthesized after *Rhodococcus* codon preference optimization. Using the plasmid in the best mutant (TH3/M4N1–59) as the object, the recombinant plasmid pNV-*sigA*$^{M4N1-59}$-*parDE*^{M} (Fig. 5a) was constructed and transformed into *R. ruber* TH3. The recombinant thus generated, TH3/pNV-*sigA*$^{M4N1-59}$-*parDE*^{M} (hereafter, TH3/M4N1–59DE^{M}), was subjected to plasmid stability evaluation using TH3/M4N1–59 as a control (Fig. 5b).

As shown in Fig. 5b, ~25 % of plasmid pNV18-*sigA*$^{M4N1-59}$ remained after four subcultures under antibiotic-free conditions, revealing that the stability of pNV18-*sigA*$^{M4N1-59}$ is poor in the *Rhodococcus ruber* host. In contrast, ~80 % of plasmid pNV-*sigA*$^{M4N1-59}$-*parDE*^{M} remained after 12 subcultures, indicating that *parDE*^{M} expression significantly enhanced plasmid stability in TH3. Finally, strain TH3/M4N1-59DE^{M} was designated the engineered strain with the most promise for industrial application.

Utilization of the TH3/M4N1-59DE^{M} cell catalyst in acrylonitrile hydration

Using TH3 as a control, the growth, nitrile hydratase activity, and catalysis performance of TH3/M4N1-59DE^{M} were evaluated. This strain exhibited a nitrile hydratase activity of 4,764 U/ml after 48 h culture, a 39.5 % increase over that of TH3. No obvious difference in cell density was observed. After the addition of an identical amount of TH3/M4N1–59DE^{M} and TH3 cells to the mimic-bioreactor containing the same volume of water, acrylonitrile was fed continuously and transformed immediately into acrylamide, a reaction catalyzed by nitrile hydratase. When the acrylamide concentration reached 300 g/l, the first batch of catalysis ended (Fig. 6a), and cells were recovered using a hollow-fiber membrane for use in the second reaction batch (Fig. 6b). As shown in Fig. 6a, the new strain exhibited a higher catalysis rate than TH3 in the batch 1 reaction. The new strain accumulated 362.5 g/l acrylamide in the batch 2 reaction, which was a 36.8 % increase over that of TH3 (Fig. 6b). The acrylamide productivity of the batch 1 and 2 reactions catalyzed by TH3/M4N1-59DE^{M} cells was 2.15 and 1.21 g/l·min, representing a 27.8 and 37.5 % increase over those of TH3, respectively. These data indicate that TH3/M4N1-59DE^{M} cells catalyze the production of higher acrylamide concentrations in one batch, and/or can be reused in many more batches.

Discussion

Acrylamide is an important chemical. Microbial bioproduction of acrylamide using in-cell nitrile hydratase as a catalyst is a successful example of industrial biocatalysis. The *SigA* gene, which encodes the sigma 70 factor, was cloned from the nitrile hydratase-producing *R. ruber* TH. Random mutation was performed to construct a *sigA* mutant library in the *amiE*-negative *R. ruber* TH3. To achieve improved acrylamide and nitrile tolerance, lethal screening and fermentation re-screening were carried out,

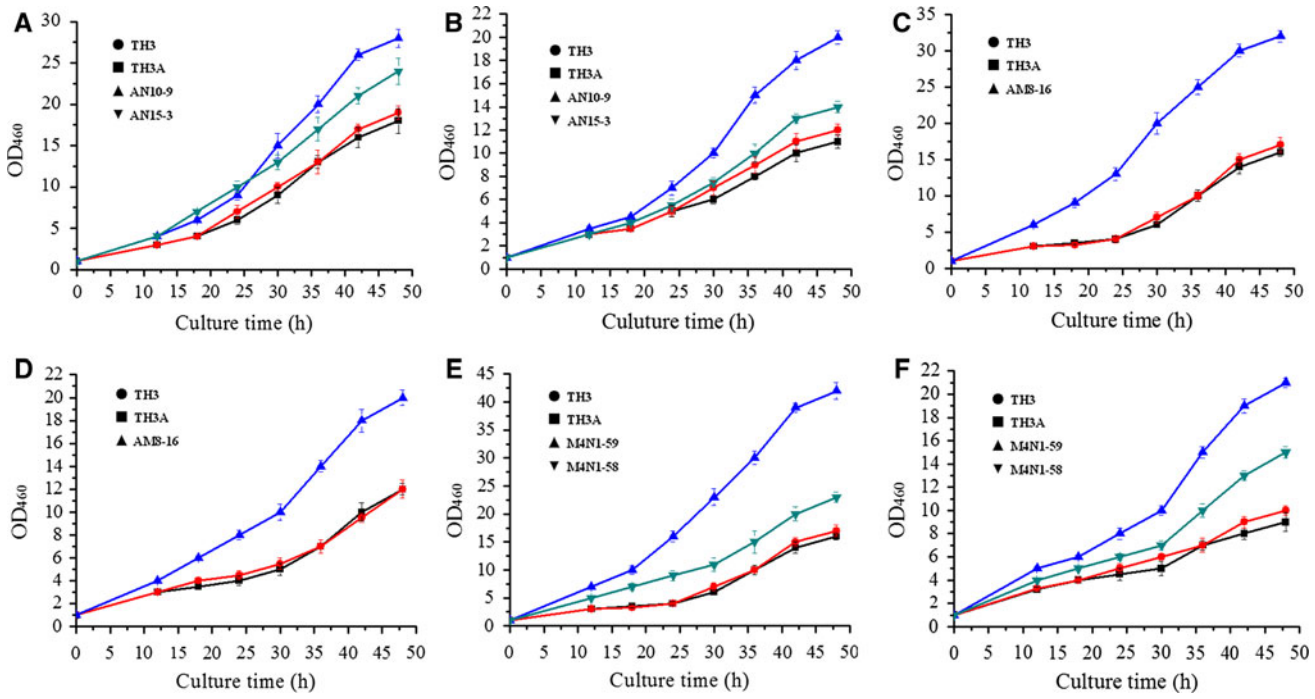


Fig. 3 Growth curves of mutant strains AN10-9 and AN15-3 under 0.1 % and 0.15 % acrylonitrile (**a** and **b**); AM8-16 and AM8-24 under 0.4 and 0.6 % acrylamide (**c** and **d**); and M4N1-48 and M4N1-59 under 0.1 % acrylonitrile and 0.6 % acrylamide (**e** and **f**), stresses, respectively

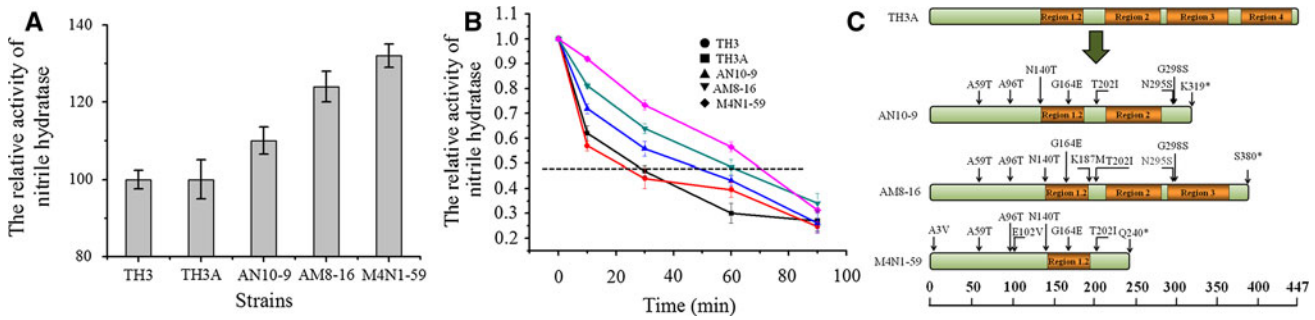
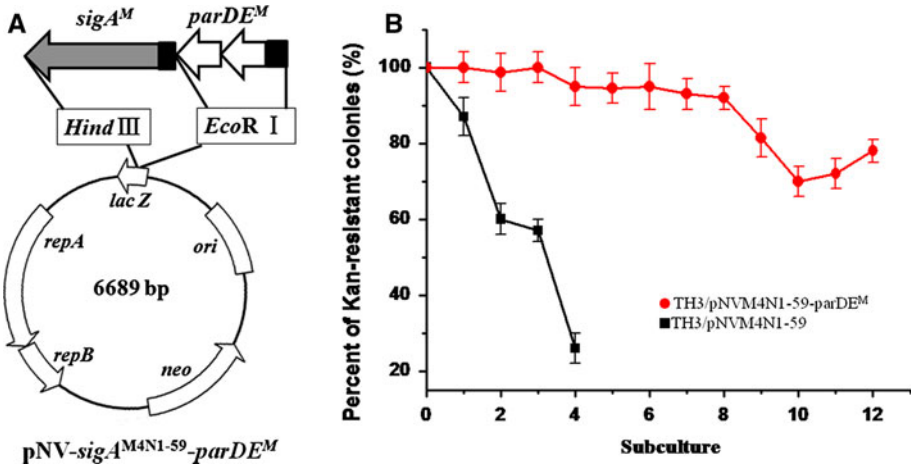


Fig. 4 Relative nitrile hydratase activity of the best mutants after culture for 48 h (**a**); relative nitrile hydratase deactivation rate of the best mutants after exposure to 40 % acrylamide (**b**); and the location of SigA mutations in the best clones, AN10-9, AM8-16, and M4N1-59, with respect to the native SigA in *R. ruber* (**c**)

Fig. 5 Relative location of the $sigA^{M4N1-59}$ and $parDE^{M}$ genes in the recombinant plasmid pNVM4N1-59-$parDE^{M}$ (**a**); stability of pNVM4N1-59-$parDE^{M}$ compared to pNVM4N1-59 in *R. ruber* TH3 (**b**). Percentage of kanamycin-resistant cells carrying the pM4N1-59-$parDE^{M}$ or pM4N1-59 plasmids, respectively, compared to the total number of the corresponding cells under kanamycin-free conditions in each subculture



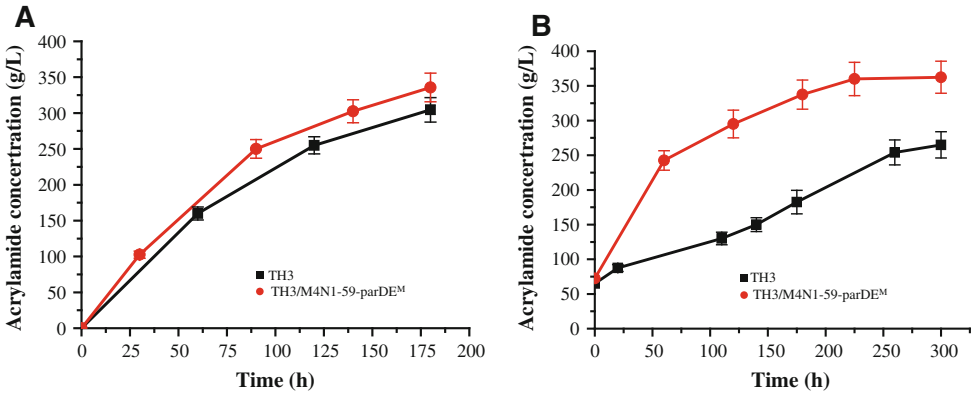


Fig. 6 Comparison of the hydration reaction efficiency of the TH3/M4N1-59DE^{M} and TH3 cell catalysts. Acrylamide accumulation curve in the batch 1 reaction. The reaction was stopped when the acrylamide concentration reached 300 g/l (**a**); acrylamide accumulation curve of a 5 h batch 2 reaction (**b**)

resulting in the TH3/M4N1-59 mutant, which exhibited the most appropriate characteristics. The *Pseudomonas* sp. RP4 *parDE* gene, responsible for plasmid stability, was codon-optimized and introduced to TH3 to enhance plasmid stability.

Compared to *R. ruber* TH3, the superior mutant TH3/M4N1-59DE^{M} showed higher nitrile hydratase activity, a higher acrylamide tolerance, a faster nitrile hydratase reaction rate, and higher acrylamide production, especially in the batch 2 bioreaction.

During transcription in bacteria, the σ factor facilitates the identification of promoters and initiates transcription to achieve specific gene expression [10]. Due to significant differences in structure and function, the σ factors identified in microbes can be grouped into diverse classes. The σ^{70} (RpoD in *E. coli*), a key sigma factor, recognizes -35 (TTGACA) and -10 (TATAAT) consensus sequences [5, 8]. The σ^{70}-RNA polymerase holoenzyme can spontaneously form transcriptionally proficient open promoter complexes to regulate the transcription of more than 1,000 genes [20]. So, multiple genes would likely be modified simultaneously upon introduction of the mutant σ^{70} factor into *R. ruber* TH3, resulting in the improved-tolerance phenotype.

Tolerance is a key phenotype for cell catalysts, which are used widely for the bioproduction of many valuable chemicals. This work revealed a novel, feasible method for increasing the bioconversion productivity of target chemicals through *sigA* mutation of the cell catalyst.

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