Chapter 2
Minimum Genome Factories in *Schizosaccharomyces pombe*

Hiromichi Kumagai, Mayumi Sasaki, Alimjan Idiris, and Hideki Tohda

Abstract This chapter gives an overview of the "minimum genome factory" (MGF) of the fission yeast *Schizosaccharomyces pombe* (*S. pombe*). The *S. pombe* genome is one of the smallest found in free-living eukaryotes. We engineered a reduction in the number of *S. pombe* genes using a large-scale gene deletion method called the LATOUR method. This method enabled us to identify the minimum gene set required for growth under laboratory conditions. The genome-reduced strain has four deleted regions: 168.4 kb of the left arm of chromosome I; 155.4 kb of the right arm of chromosome I; 211.7 kb of the left arm of chromosome II; and 121.6 kb of the right arm of chromosome II. These changes represent a loss of 223 genes of an estimated 5,100. The 657.3-kb deletion strain was less efficient at taking up glucose and some amino acids from the growth media than the parental strain. This strain also showed increased gene expression of the mating pheromone M-factor precursor and NADP-specific glutamate dehydrogenase. There was also a 2.7-fold increase in the concentration of cellular ATP, whereas levels of heterologously produced proteins, such as the green fluorescent protein and the secreted human growth hormone, increased by 1.7 fold and 1.8 fold, respectively.

Keywords LATOUR method • Minimum genome factory • Protein expression system • *Schizosaccharomyces pombe* (*S. pombe*)

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2.1 *Schizosaccharomyces pombe*

New pathways for designing microorganisms for industrial-scale production of biological materials have been made possible by significant advances in genome sequencing, bioinformatics, and genetic engineering. The so-called minimum genome factory (MGF) has been created in which unnecessary or detrimental genes are deleted from the microorganism, leaving only the genes necessary for industrial production of the desired molecule. The first examples of MGFs are *Escherichia coli*, *Bacillus subtilis*, and *Schizosaccharomyces pombe* (*S. pombe*) (Ara et al. 2007; Giga-Hama et al. 2007; Fujio 2007; Mizoguchi et al. 2007). This chapter provides an overview of MGFs in *S. pombe*.

S. pombe is a unicellular eukaryote belonging to the Ascomycetes class of fungi. Although *S. pombe* belongs to the same class as the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), it is taxonomically and evolutionarily distant. *S. pombe* reproduces by fission, a process similar to that used by higher eukaryotic cells. Furthermore, *S. pombe* shares many molecular, genetic, and biochemical features with multicellular organisms, making it a particularly useful model for studying the function and regulation of genes from more complex species (Zhao and Lieberman 1995). These attributes make it attractive for industrial fermentation, a process characterized by high cell densities, short fermentation times, and the use of chemically defined media lacking components derived from animal cells. Thus, *S. pombe* is a useful host for heterologous expression of molecules derived particularly from higher organisms.

Figure 2.1 shows the MGF concept. Microorganisms have a variety of genes that are expressed during adaptation to different environmental conditions. These genes are thought to be unnecessary under nutrient-rich growth conditions. Hence, a minimal gene set is required for cellular viability, and identifying this gene set would provide important clues about the evolutionary origins of eukaryotic organisms. In addition, minimal gene sets can be used to construct minimal genome factories for industrial production of biological materials.

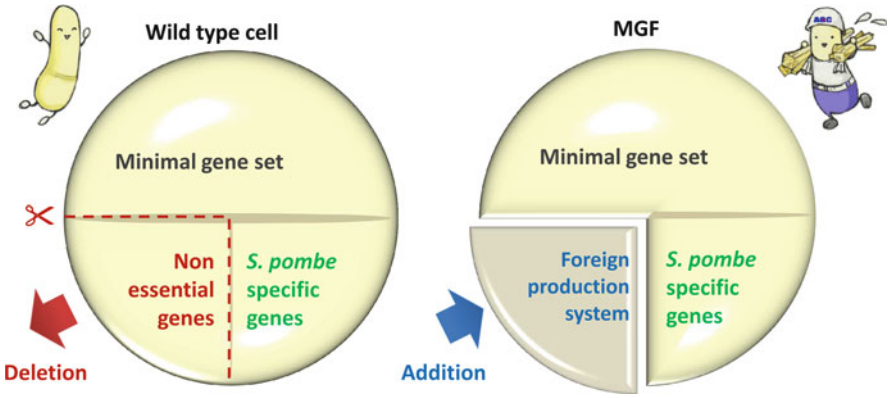

Fig. 2.1 Concept of minimum genome factory

Development of microbial production systems requires computer modeling and simulation of cellular metabolic systems to optimize metabolic networks (Medema et al. 2012). However, understanding the metabolic systems that are predicted to be present in microorganisms can be problematic, as complex intracellular metabolic pathways often interfere with experiments designed to elucidate such systems. Recently, reducing the genome sizes of some microorganisms has been employed as a strategy to simplify their intracellular metabolic pathways while maintaining their growth efficiencies. For successful construction of an MGF, it has been proposed that effective use of intracellular energy can be achieved by eliminating unnecessary genes (Fujio 2007). Comparative genomics supports this hypothesis, and it has been speculated that genome reduction can be a selective process favoring adaptation to low-nutrient environments for effective energy utilization (Moya et al. 2009).

Several genome-reduced microorganisms are reported to have beneficial properties. In *E. coli*, for example, such benefits include improved electroporation efficiency, accurate propagation of recombinant genes and unstable plasmids (Posfai et al. 2006), and an increase in L-threonine production (Mizoguchi et al. 2008; Baba et al. 2006). In *B. subtilis*, increases in production and secretion levels of heterologous enzymes have also been noted following genome reduction (Manabe et al. 2011; Morimoto et al. 2008; Kobayashi et al. 2003). Deletion of part of the *S. cerevisiae* genome, which results in altered carbon metabolism, has also been reported (Murakami et al. 2007).

The *S. pombe* genome sequencing project was completed in 2002 (Wood et al. 2002). The whole genome, which is distributed on three chromosomes, is estimated at 13.8 Mb. In *S. pombe*, a single systematic genome-wide collection of gene deletion mutants has been reported (Kim et al. 2010), and a pilot study reported deleting 100 of its genes (Decottignies et al. 2003). Essential genes in *S. pombe* constitute about 25 % of its total gene content (1,260/5,100), which is a higher percentage than that found in other model organisms because *S. pombe* has one of the smallest gene numbers found among free-living eukaryotes.

Genetic manipulation of *S. pombe* is well established. Hence, we investigated the minimal gene set required by this free-living model eukaryote by deleting as many nonessential genes as possible. From this, we generated *S. pombe* mutants dedicated to heterologous protein production (Sasaki et al. 2013).

2.2 Construction of Genome-Reduced Fission Yeast Strains

We have developed a unique method for chromosomal modification in *S. pombe*. This method, which we have designated the "latency to universal rescue method" (LATOUR method; Fig. 2.2), is an extremely simple method that only requires a negative selectable marker for its application. No foreign sequences remain following chromosomal modification. Using this method, it was easy to delete a chromosomal segment of more than 100 kb containing 33 genes at once (Hirashima et al. 2006). LATOUR is a very useful method for construction of genome-reduced strains and for clean deletion of unnecessary genes. It is also possible to identify genes that are essential in *S. pombe* by this method.

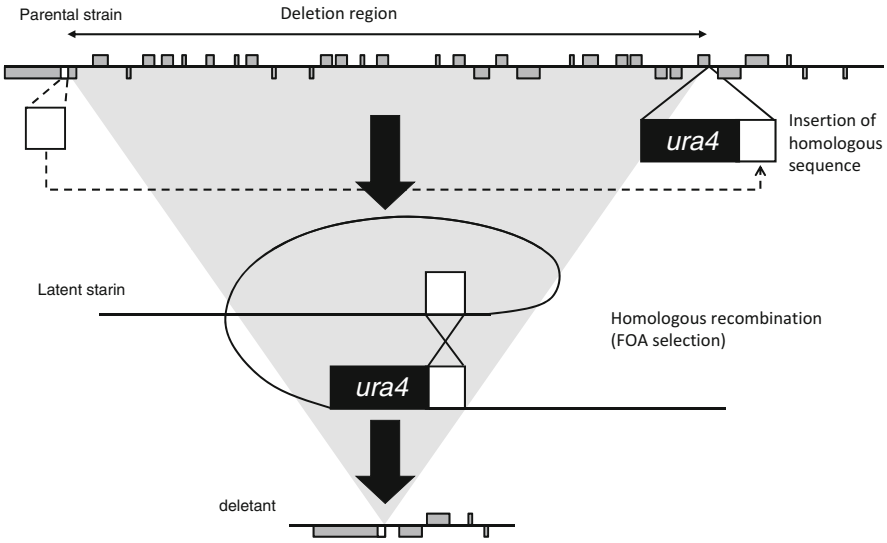

Fig. 2.2 Schematic representation of the deletion by the LATOUR method. The portion including the *ura4* sequence is the introduced modification fragment for homologous recombination. The direct repeats are not contained in the modification fragment, and the *ura4* and target genes are places between direct repeats on the chromosome during the latent stage. The important difference from previous methods is that the target gene to be deleted is retained during the stage in which the modification fragment is introduced

The *S. pombe* genome was reduced by deletion of the terminal regions of chromosomes I and II using the LATOUR method. This method generated a 657.3-kb (5.2 % of the total 12.57-Mb genome size sequenced to date) deletion strain that maintained its growth rate. The genome-reduced strain (called the quadruple-deletion strain) has four deleted genomic regions: 168.4 kb of the left arm of chromosome I; 155.4 kb of the right arm of chromosome I; 211.7 kb of the left arm of chromosome II; and 121.6 kb of the right arm of chromosome II. These deletions correspond to a loss of some 223 genes from the original 5,100 that are estimated to be present in *S. pombe* so far (Sasaki et al. 2013).

Figure 2.3 summarizes the reduced genome size and gene number for this strain. The gene number of the quadruple-deletion strain is currently the smallest among eukaryotic model organisms. The genes that were deleted are summarized in Table 2.1.

We determined that the most-terminal essential genes in the left and right arms of chromosomes I and II were *trs33* (ALT, left arm of chromosome I), *sec16* (ART, right arm of chromosome I), *zas1* (BLT, left arm of chromosome II), and *usp109* (BRT, right arm of chromosome II) (Fig. 2.3). Although it has been reported that the genes SPAC1F8.07c (ALT), SPBC1348.06c (BLT), *alr2* (BLT), and *dea2* (BLT), which are located on the telomeric side of *trs33* and *zas1*, are also essential for growth (Kim et al. 2010), a deletion strain that does not include the genes SPBC1348.06c, *alr2*, or *dea2* exhibited no decrease in growth rate in comparison to

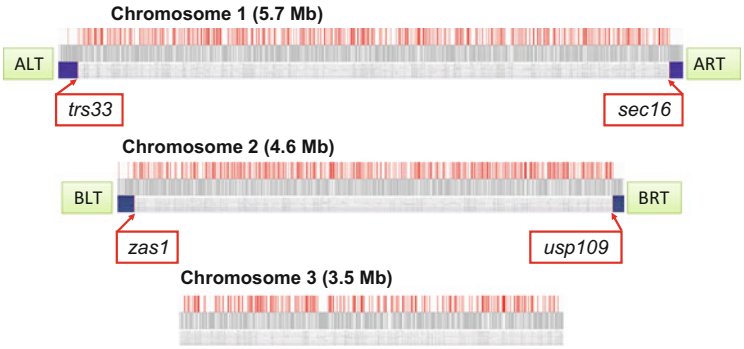

Fig. 2.3 Deletion regions of *Schizosaccharomyces pombe* chromosome. *Red box*, essential gene; *gray*, nonessential; *blue*, deletion region in the quadruple-deletion strain

Table 2.1 Classification of deleted genes in the quadruple-deletion strain

Term	Name	ALT	ART	BLT	BRT	Subtotal
GO:0055085	Transmembrane transport	8	10	20	3	41
GO:0006520	Cellular amino-acid metabolic process	4	5	4	1	14
GO:0006091	Generation of precursor metabolites and energy	3	3	2	0	8
GO:0006486	Protein glycosylation	3	0	0	4	7
GO:0005975	Carbohydrate metabolic process	0	2	2	2	6
GO:0007155	Cell adhesion	2	1	2	1	6
GO:0006355	Regulation of transcription, DNA dependent	2	0	4	0	6
GO:0006310	DNA recombination	2	0	0	1	3
GO:0006766	Vitamin metabolic process	2	0	0	1	3
GO:0006629	Lipid metabolic process	1	0	1	0	2
GO:0007126	Meiosis	2	0	0	0	2
GO:0055086	Nucleobase-containing small molecule metabolic process	0	0	2	0	2
GO:0006351	Transcription, DNA dependent	1	1	0	0	2
GO:0006260	DNA replication	0	0	0	1	1
GO:0070882	Cellular cell wall organization or biogenesis	0	1	0	0	1
GO:0007059	Chromosome segregation	1	0	0	0	1
GO:0051186	Cofactor metabolic process	0	0	1	0	1
GO:0000747	Conjugation with cellular fusion	0	1	0	0	1
GO:0007010	Cytoskeleton organization	0	0	0	1	1
GO:0071941	Nitrogen cycle metabolic process	0	0	0	1	1
GO:0006605	Protein targeting	0	1	0	0	1
GO:0023052	Signaling	0	1	0	0	1
	Others	23	22	16	13	74
	Conserved unknown	5	1	2	3	11
	Sequence orphan	6	1	3	3	13
	Pseudogene	5	0	4	4	13
	Dubious	0	1	0	0	1
	Total deletion gene	70	51	63	39	223

that of the parental strain. Whether this phenotype is a unique property of our laboratory strain is not known.

The quadruple-deletion strain showed a slight decrease in growth rate and smaller cell size in comparison to the parental strain. This reduced cell size may be similar to what is observed when cells are subject to nutrient stress (Fantes and Nurse 1977). An increase in the expression levels of nitrogen starvation-response genes was observed in the quadruple-deletion strain during its logarithmic growth phase. Expression of the mating pheromone M-factor precursor increased in this strain. We postulate that mimicking of the nitrogen starvation response in the quadruple-deletion strain is related to downregulation of TORC1 activity (Matsuo et al. 2007).

The glucose uptake efficiency, microarray analysis, and metabolome analysis in the quadruple-deletion strain were compared against a nonauxotrophic strain. The quadruple-deletion strain showed the following characteristics:

1. Slightly decreased glucose uptake and ethanol production
2. Decreased amino-acid uptake
3. Induction of sexual development genes and genes associated with nonglucose metabolism
4. Increased ATP concentration

2.3 *Schizosaccharomyces pombe* MGF Mutants as Hosts for Recombinant Protein Expression

We compared the production levels of various recombinant proteins using the genome reduced strains and compared them to those of the parental strain (Sasaki et al. 2013). We constructed vectors for heterologous expression of enhanced green fluorescent protein (EGFP), human transferrin, or human growth hormone, and integrated these vectors into the parental and the genome-reduced strains. Next, GFP fluorescence in each of the deletion mutants (per milliliter or cell numbers) was measured. Surprisingly, the expression levels, which were higher in the deletion mutants during all the growth phases, depended on the length of the deletion (i.e., from short to long regions). In the case of EGFP expression, the strain showing the highest productivity was the quadruple-deletion strain. The EGFP production rate in the quadruple-deletion strain increased about 1.7 fold in comparison to that of the parental strain during the logarithmic-phase growth. Based on transcriptome and metabolome analysis, we speculate that the increased EGFP expression in the deletion mutant could be related to an increase in intracellular GTP (which is required for ribosomal activity), thereby leading to increases in translation and EGFP production. Increased intracellular ATP levels could also lead to activation of amino-acid biosynthesis reactions. However, an increase in the concentration of intracellular amino acids was not observed, and some intracellular amino acids in the quadruple-deletion strain decreased. Our results also showed that the expression levels for human growth hormone and human transferrin reached much higher levels in the genome-reduced strains than in the parental strain. Taken together, these results

suggest that genome reduction is a valuable tool for construction of heterologous protein production systems.

The LATOUR method is a powerful tool for clean deletion of protease-encoding genes that are a problem during expression and purification of recombinant proteins. As reported previously (Idiris et al. 2006, 2009, 2010), multi-protease gene deletion mutants are useful for producing recombinant proteins that are particularly sensitive to proteases. One such mutant, which we called A8, was constructed by multiply disrupting eight protease genes known to cause protein degradation by using conventional gene deletion technology. Each of the eight target genes was disrupted by substitution with the *ura4*$^{+}$ selectable marker gene. As a result, eight *ura4*$^{-}$ sequences were retained in the A8 strain where the eight protease genes were originally located. However, the remaining *ura4*$^{-}$ genes could prevent the *ura4*$^{+}$ marker gene being used for further gene deletion or integration experiments. Consequently, we have recently reconstructed the A8 strain using the LATOUR method without retention of any of the *ura4* sequences in the genome.

2.4 Conclusions

We have shown that the MGF approach, in which a parental line has been engineered to contain the minimum number of genes necessary for growth and survival via inactivation of unnecessary or detrimental genes, is effective at creating new lines that can be used to produce a variety of heterologous proteins. The production levels of recombinant EGFP, human transferrin, and human growth hormone in the genome-reduced strains were higher than in the parental strain. These results confirm that the gene deletion technology we have developed is very useful for constructing *S. pombe* mutants for recombinant protein production.

Although much progress has been made in manufacturing recombinant proteins, many issues still need to be solved. The genome-reduced strain is suitable for modeling and simulation of cellular metabolism. However, problems related to system unpredictability are often caused by the complex nature of the intracellular metabolic system, because not all the cellular metabolic intermediates have been completely elucidated. A simplified cellular metabolism, while maintaining satisfactory growth and requisite functions (e.g., high recombinant protein productivity), should lead to successful control of intracellular metabolism in accordance with the metabolic modeling/simulations and thus benefit high-level production of biological materials.

The MGF concept should help advance heterologous protein production systems, whole-cell biocatalysts, and synthetic processes for small molecules. The continued development of MGF technology promises to shed light on issues currently limiting large-scale industrial production of biological materials.

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