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Review

Studies on Application of Ion Beam Breeding to Industrial Microorganisms at TIARA

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Abstract: Mutation-breeding technologies are useful tools for the development of new biological resources in plants and microorganisms. In Takasaki Ion Accelerators for Advanced Radiation Application (TIARA) at the National Institutes for Quantum and Radiological Science and Technology, Japan, ion beams were explored as novel mutagens. The mutagenic effects of various ion beams on eukaryotic and prokaryotic microorganisms were described and their application in breeding technology for industrial microorganisms were discussed. Generally, the relative biological effectiveness (RBE) depended on the liner energy transfer (LET) and the highest RBE values were obtained with $^{12}C^{5+}$ ion beams. The highest mutation frequencies were obtained at radiation doses that gave 1%–10% of surviving fraction. By using $^{12}C^{5+}$ ion beams in this dose range, many microorganisms have been improved successfully at TIARA. Therefore, ion-beam breeding technology for microorganisms will have applications in many industries, including stable food production, sustainable agriculture, environmental conservation, and development of energy resources in the near future. Moreover, genome analyses of the ion-beam-induced mutants are in progress to clear the differences of mutational functions induced by different LET radiations in microorganisms. Further characterizations of mutations induced by different LET radiations will facilitate more effective use of ion beams in microorganisms breeding.

Keywords: ion beam; microorganism; mutagenesis; mutation breeding; TIARA

1. Introduction

The growth of the global population increases stable food production in sustainable environmental conditions. It is necessary to expand the variety of available biological resources with functions such as environmental tolerance. Mutation breeding is an applicable technology for expanding variations of biological resources. In plant breeding, >3200 mutant varieties have been generated and registered in the Joint FAO/IAEA Mutant Variety Database [1]. The Joint FAO/IAEA was established by the Food and Agriculture Organization of the United Nations (FAO) and the International Atomic Energy Agency (IAEA). Most mutant varieties were generated by using ionizing radiation, chiefly gamma rays. However, recently, ion beams have been used as novel mutagens, and the number of mutants in the database generated by using ion beams is increasing.

Takasaki Ion Accelerators for Advanced Radiation Application (TIARA), which was established at Takasaki Radiation Research Institute (TARRI), National Institutes for Quantum and Radiological Science and Technology (QST), Takasaki, Japan, is an ion irradiation facility designed to use the features of ion beams for advanced science such as material science and biotechnology. Since the 1990s, QST has been conducting basic research on the induction of mutation in plants using ion beams accelerated by an azimuthally varying field (AVF) cyclotron at TIARA. The AVF cyclotron at TIARA

can provide various ion beams from 5 MeV $^{1}H^{+}$ to 500 MeV $^{197}Au^{31+}$ ions and is equipped with ten horizontal beamlines and four vertical branch beamlines [2]. For ion-beams breeding, the ion beams are transported to the irradiation device through a vertical beamline (Figure 1). The vertical beamlines are helpful to irradiate unfixed samples such as tissue culture materials. For uniformly irradiation, ion beams accelerated by the AVF cyclotron were scanned on a 60×60 mm^2 area in a vacuum chamber and exited through a 30-μm titanium foil into atmospheric conditions. Target samples were put onto a Petri dish and covered with 7.5 μm of kapton polyimide film were irradiated with ion beams at room temperature in the atmospheric conditions (Figure 1C). In research into *Arabidopsis thaliana*, a widely used model plant, UV-resistant mutants [3], UV-sensitive mutants [4], and mutants in flavonoid transport [5] and in auxin regulation [6] have been successfully generated by ion-beam mutagenesis. The generation of these mutant resources has greatly contributed to understanding the mechanisms of UV response, pigment accumulation, and hormone responses in plants. The biological effects of ion beams, which are high linear energy transfer (LET) radiations, are generally greater than those of low-LET radiation such as gamma rays and electrons [7,8]. For instance, the mutation frequency induced in *A. thaliana* by carbon ion beam irradiation was higher than that by electrons [9,10]. Therefore, this technology has been applied in horticulture to generate new varieties of ornamental plants. New mutant varieties with such characteristics as flower colors and shapes have been generated in carnation [11], chrysanthemum [12], petunia [13], osteospermum [14], and these novel garden varieties are available commercially. Ion-beam breeding has also been used in the field of sustainable agriculture and applied to generate low-temperature-tolerant netted melons [15], blast-resistant mutants of rice [16], rice mutants that grown well even in low fertilizer conditions [17], and low-cadmium rice [18]. Since the early 1990s, when mutation induction by ion beams was performed for the first time at TIARA, numerous mutagenesis experiments using ion beams have been conducted in higher plants. The characteristics of the mutations induced and mutant varieties generated by ion beams are well-documented in higher plants [19].

Microorganisms are closely associated with human life; for example, they are frequently used for the production of foods and medicines. They are used in a wide range of industries as they have various capabilities to produce, decompose, and accumulate complex chemicals. Although the ion-beam mutation technology has mainly been used for plant breeding, it is also useful for breeding of industrial microorganisms. In QST, we have been working with many collaborators to develop ion-beam technology for generating useful microbial resources that can be used in fields such as agriculture, fermentation, and environmental conservation. This review summarizes our work on ion-beam mutation technology for microorganisms conducted at TIARA.

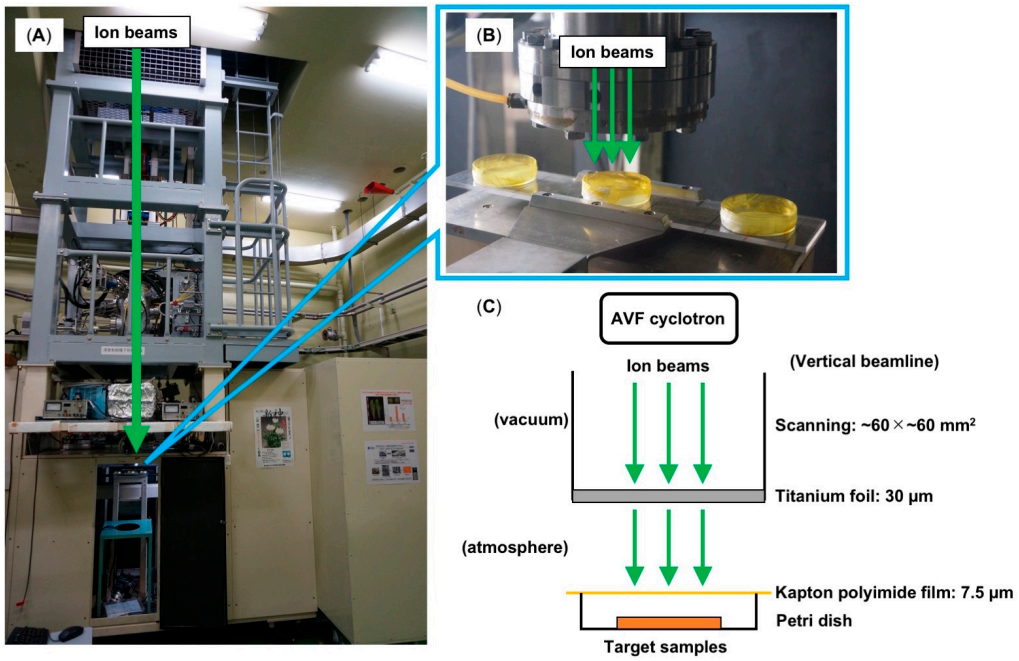

Figure 1. Outline of the ion beams irradiation. (**A**) Photo of the irradiation device for ion-beam breeding. (**B**) A magnified view of the irradiation area and target samples. (**C**) Schematic diagram of ion-beam irradiation. The ion beams were accelerated by the azimuthally varying field (AVF) cyclotron and transported to a vertical beamline. Next, the accelerated ion beams scanned the irradiation area ($60 \times 60\,mm^2$) uniformly in a vacuum chamber and exited through a 30-μm titanium foil to atmospheric conditions. Finally, the target microorganism samples were irradiated with ion beams at room temperature in the atmospheric conditions.

2. Mutagenic Effects of Radiations with Different LET in Microorganisms

Like plants, industrial microorganisms also have been bred to have beneficial properties by conventional mutation methods, mainly using chemicals (ethyl methanesulfonate, methyl methanesulfonate, and N-methyl-N'-nitro-N-nitrosoguanidine), UV, X-rays, and gamma rays as mutagens. However, acquisitions of desired mutants by conventional methods are relatively difficult because of low mutation rates and/or a multitude of mutations. Thus, it takes much time and labor to acquire useful mutants via conventional methods, and it was thus essential to develop a new method for microorganism mutation that could more effectively induce mutations. Ion beams characteristically have high LET and cause high density DNA damage (i.e., clustered damage). In mammalian cells and plants, the relative biological effectiveness (RBE) of ion beams depends on the LET and peaks in the range of LET from about 100 to 400 keV/μm [19–21]. In plants, high-LET ion beams with high RBE can induce different characteristics of mutagenic effects compared with low-LET gamma rays [19]. Therefore, we have been interested in using ion beams for mutation breeding of microorganisms as they could be more effective mutagens than traditional approaches. However, since little was known about the mutagenic effects of ion beams on microorganisms, an investigation on the characteristics of mutations induced by different LET radiations in microorganisms was performed. In most of experiments, five kinds of ion beams ($^{4}He^{2+}$, $^{12}C^{5+}$, $^{12}C^{6+}$, $^{20}Ne^{8+}$, and $^{40}Ar^{13+}$) accelerated by an AVF cyclotron at TIARA were used to irradiate microorganism samples in the atmospheric conditions (Table 1). In all experiments, the prepared microorganism samples that were fixed on a cellulose membrane or agar and put onto a Petri dish were irradiated. At the target surface, the energy of the ion beams passing through the atmosphere and kapton film slightly decreased (Table 1). The penetration range and LET of ion beams were calculated using the IRAC M code [22]. The irradiated dose for microorganism was calculated from the formula: Dose (Gy) = particle fluence ($/cm^2$) × LET (keV/μm) × sample density (g/cm^3) × 1.6×10^{-9}. The fluence was calculated from the real-time simultaneous

measurement value of electric current. For this dose calculation, sample density was expedientially assumed to be 1 g/cm^3. Although the projectile ranges of these ion beams in water are as short as several millimeters, but sufficient to irradiate thin samples such as microorganism cells. Although the projectile ranges of these ion beams in water are as short as several millimeters, but sufficient to irradiate thin samples such as microorganism cells.

Table 1. Properties of ion beams used for mutation breeding at the National Institutes for Quantum and Radiological Science and Technology (QST).

Ion	Total Accelerated Energy (MeV)	Total Energy at Target Surface (MeV)	LET at Target Surface (keV/μm)	Penetration Range in Water (μm)
$^{4}He^{2+}$	50	48	16	1670
$^{12}C^{6+}$	320	311	76	2270
$^{12}C^{5+}$	220	208	107	1110
$^{20}Ne^{8+}$	350	316	317	600
$^{40}Ar^{13+}$	460	310	1550	150

Aspergillus oryzae, known as koji mold, is an industrially important microorganism widely used for the production of traditional Japanese fermented foods and beverages such as miso, soy sauce, and sake, as well as for the production of various enzymes used in the industry [23]. To evaluate the effect of ion beams on this filamentous fungus, the lethal effects of different LET radiations on freeze-dried conidia of *A. oryzae* onto the cellulose membranes were investigated, based on the dose required to reduce the surviving fraction to 10% (D_{10}). The RBE was calculated by the D_{10} value of ion beams divided by the D_{10} value of gamma rays. The most effective ionizing radiation in terms of lethal effect among the different LET radiations was the $^{12}C^{5+}$ ion beam (107 keV/μm), and its RBE value was 4.7. Like other organisms, the RBE for *A. oryzae* was shown to be dependent on the LET (Figure 2).

Selenate is toxic analog of sulfate, forms selenomethionine by incorporation during the biosynthesis of methionine, and inhibits cell growth. Selenate resistance in *A. oryzae* is mainly caused by the loss of function of the sulfate permease gene (*sB*) and/or the ATP sulfurylase gene (*sC*). Mutagenic effects have been evaluated by monitoring the frequency of selenate resistant mutants (Figure 3). The $^{12}C^{6+}$ ion beams were the most effective in inducing selenate resistant mutants among three radiations with different LETs. The highest value of mutant frequency (3.47×10^{-3}) for high-LET $^{12}C^{6+}$ ion beams was obtained at a dose of 700 Gy, and it was 1.8 times that of low-LET gamma rays (1.93×10^{-3} at 1400 Gy; Table 2). The highest value of the mutant frequency with $^{12}C^{5+}$ ion beams (1.67×10^{-3} at 400 Gy) was slightly lower than that with the other two types of radiation. These highest values of mutant frequency were observed in a dose range that gave a surviving fraction of 0.1% to 1% for each radiation (Figure 3). The $^{12}C^{5+}$ ion beams were the most effective in mutation frequency at low doses (200 to 400 Gy). To determine the mutation sites in selenate resistant mutants that were induced by $^{12}C^{5+}$ ion beams or gamma rays, direct DNA sequencing of genomic PCR products was carried out. The average numbers of mutations per clone in *sB* and *sC* for gamma rays (5.26 and 2.06) were four and 1.9 times those generated with $^{12}C^{5+}$ ion beams (1.30 and 1.09), respectively, indicating that gamma rays tended to generate a multitude of mutations in the same locus of the same clone. In genomic PCR analysis, DNA fragments of either *sB* or *sC* loci could not be amplified in 71.25% and 48.89% of selenate resistant mutants generated by gamma-rays and $^{12}C^{5+}$ ion beams, respectively. These failures of the genomic PCR amplification may be attributed to large-scale variations, such as large DNA deletions, translocations, and inversions. Comparative genomic hybridization analysis of a selenate resistant mutant induced by $^{12}C^{5+}$ ion beams revealed large deletions (2 and 340 kb). In addition, chromosomal rearrangements by translocation were observed in mutants induced by gamma rays and ion beams (Table 3) [24]. Analyses suggested that high-LET $^{12}C^{5+}$ ion beams as well as low-LET gamma rays induced base substitutions, frameshifts, deletions, and large-scale mutations in *A. oryzae*. However, some differences between $^{12}C^{5+}$ ion beams and gamma rays were observed in the frequencies of these mutations. The results obtained in this pioneering study using *A. oryzae* suggested that ion beams could be used as novel mutagens to develop useful microorganisms, as also demonstrated in plant breeding.

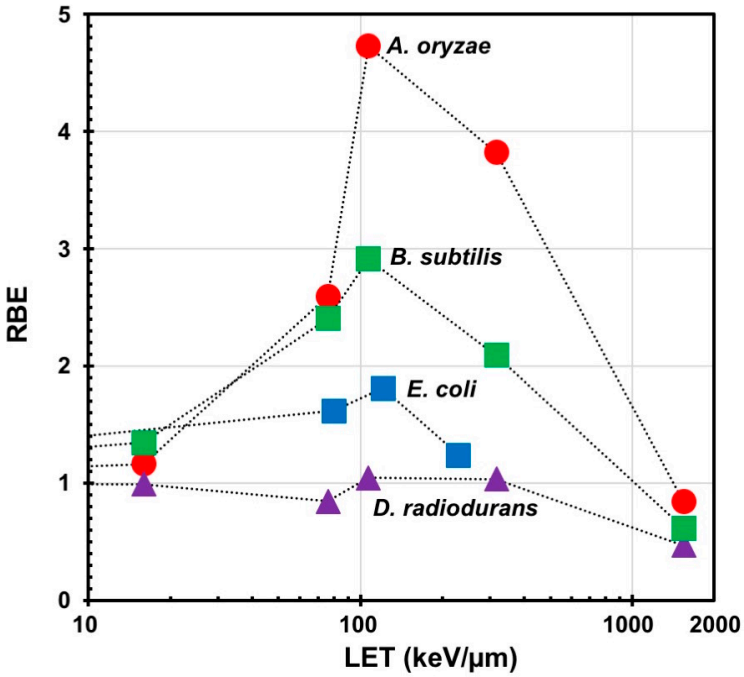

Figure 2. Relative biological effectiveness (RBE) for lethality as a function of linear energy transfer (LET). Data are plotted on the linear (RBE) and logarithmic (LET) scales. Symbols: Red circles, *Aspergillus oryzae*; blue squares, *Escherichia coli*; green squares, *Bacillus subtilis*; purple triangles, *Deinococcus radiodurans*. Data are modified from [24–27]. RBEs were recalculated based on the LET at the target surface. The highest values of RBE were obtained using 220 MeV $^{12}C^{5+}$ ion beams for which the LET is 107 keV/μm in all microorganisms tested except *D. radiodurans*, for which no RBE peak was observed because this bacterium was equally resistant to the ion beam and gamma rays.

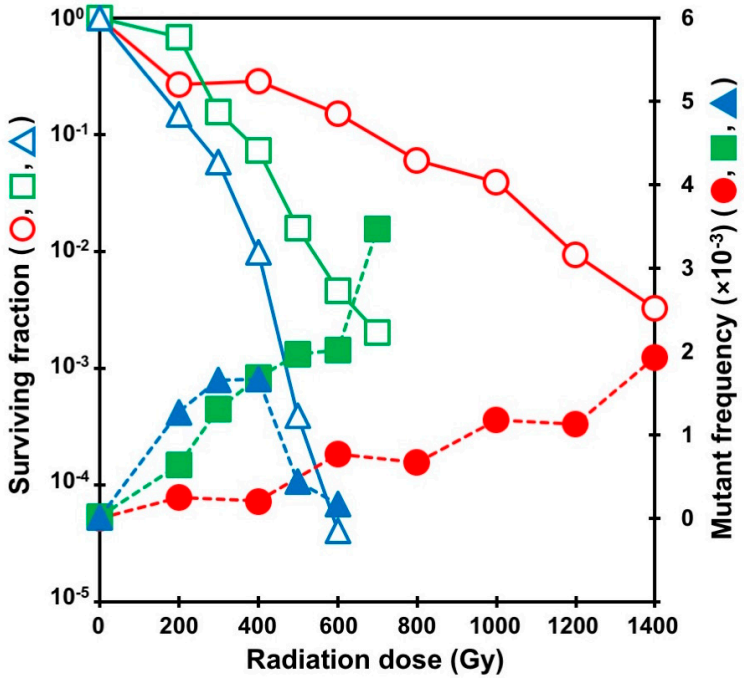

Figure 3. Survival curves and selenate-resistant mutant frequencies in *A. oryzae* exposed to gamma rays and carbon ion beams. Open and closed symbols indicate surviving fraction and mutant frequency, respectively. Symbols: Circles, gamma rays; squares, $^{12}C^{6+}$ ion beams; triangles, $^{12}C^{5+}$ ion beams. Data are modified from Toyoshima et al. (2012) [24]. The highest values of mutant frequency were obtained at a radiation dose giving a surviving fraction of around 0.1% to 1%.

Another koji mold, *Aspergillus sojae*, is also an industrially important microorganism that is used for brewing of soy sauce. Investigation of the effects of $^{4}He^{2+}$, $^{12}C^{5+}$, and $^{20}Ne^{8+}$ ion beams on wet germinated conidia of *A. sojae* onto the agar plates suggested that the $^{12}C^{5+}$ ion beams had the greatest lethal effect [28]. The lethal effect of $^{4}He^{2+}$ ion beams was comparable with that of $^{20}Ne^{8+}$ ion beams. A pyrithiamine is a toxic pyridine analog of thiamine. It inhibits the enzymatic production of thiamine pyrophosphate, an essential cofactor required for biosynthesis of thiamine, and prevents cell growth. Pyrithiamine resistance in *A. sojae* is caused by mutations of the *thiA* gene. Mutagenic effects were evaluated by monitoring the frequency of pyrithiamine resistant mutants. The highest value of mutant frequency was for $^{12}C^{5+}$ ion beams (5.60×10^{-5}), over 20 times higher than that for UV (0.26×10^{-5}), which is often used as a mutagen in conventional methods (Table 2). The highest value of mutant frequencies obtained using $^{4}He^{2+}$ and $^{12}C^{5+}$ ion beams was observed at a dose that resulted in a survival fraction of below 1%, while the dose for $^{20}Ne^{8+}$ ion beams that resulted in the highest mutant frequency was around D_{10} [28].

Table 2. Comparison of the highest mutant frequency.

Microorganism	Radiation	Dose (Gy)	Mutant Frequency	Target Gene
Fungi and Yeast				
Aspergillus oryzae	^{60}Co gamma rays	1400	1.93×10^{-3}	*sB* and *sC*
	$^{12}C^{5+}$	400	1.67×10^{-3}	
	$^{12}C^{6+}$	700	3.47×10^{-3}	
Aspergillus sojae	UV	No data	0.26×10^{-5}	*thiA*
	$^{4}He^{2+}$	300	3.56×10^{-5}	
	$^{20}Ne^{8+}$	200	1.88×10^{-5}	
	$^{12}C^{5+}$	200	5.60×10^{-5}	
Saccharomyces cerevisiae	^{60}Co gamma rays	66	0.16×10^{-5}	*ura3*
	$^{12}C^{5+}$	100	1.85×10^{-5}	
Bacteria				
Deinococcus radiodurans	^{60}Co gamma rays	8000	1.93×10^{-6}	*rpoB*
	$^{4}He^{2+}$	6000	0.92×10^{-6}	
	$^{12}C^{6+}$	6000	0.74×10^{-6}	
	$^{12}C^{5+}$	6000	1.38×10^{-6}	
	$^{20}Ne^{8+}$	8000	1.71×10^{-6}	
	$^{40}Ar^{13+}$	15,000	1.08×10^{-6}	
Rhodococcus erythropolis	$^{4}He^{2+}$	600	8.67×10^{-7}	*rpoB*
	$^{12}C^{5+}$	800	9.45×10^{-7}	

Data are modified from [24,27–30].

Table 3. Characteristics of radiation-induced mutations in *Aspergillus oryzae*.

Radiation	^{60}Co Gamma Rays	$^{12}C^{5+}$ Ion Beams
Lethal effect[*1]	1.0	4.7
Mutagenic Effect[*2]	1.93×10^{-3}	1.67×10^{-3}
A multitude of mutations[*3]	5.26, 2.06	1.30, 1.09
Large-scale mutations[*4]	71.25%	48.89%
Chromosomal rearrangement	Observed	Observed

[*1], The RBE is shown; [*2], The frequency of the selenate resistant mutant is shown; [*3], The average number of mutations per clone in *sB* and *sC* genes are shown, respectively; [*4], The frequency of failure of the genomic PCR amplification in *sB* and/or *sC* loci is shown; Data are modified from Toyoshima et al. (2012) [24].

Rhizomucor miehei is a fungus used in the industry to produce enzymes required for cheese manufacturing. Investigation of the effects of $^{4}He^{2+}$, $^{12}C^{5+}$, $^{20}Ne^{8+}$, and $^{40}Ar^{13+}$ ion beams on dried spores of *Rh. miehei* onto the Petri dishes showed that the lethal effects of $^{12}C^{5+}$ and $^{20}Ne^{8+}$ ion beams

were about 10 times higher than those of $^{4}He^{2+}$ and $^{40}Ar^{13+}$ beams [31]. $^{12}C^{5+}$ ion beams at a dose that gives a surviving fraction of around 1% were the best in terms of mutation frequency among the radiations examined [32].

The effects of radiation on the budding yeast *Saccharomyces cerevisiae*, among the most widely used model eukaryotic microorganisms, have also been investigated by irradiating filtered cells of *S. cerevisiae* with $^{12}C^{5+}$ ion beams in a dose range from 10 to 300 Gy, and gamma rays. The yeast was much more sensitive to the $^{12}C^{5+}$ ion beams than to gamma rays. 5-Fluoroorotic acid is a toxic pyrimidine analog of uracil that inhibits pyrimidine biosynthesis by interacting with orotidine-5′-phosphate decarboxylase encoded by the *ura3* gene in *S. cerevisiae*. By disrupting the *ura3* gene, 5-fluoroorotic acid-resistant mutants can be generated. Thus, to evaluate the mutagenic effects of gamma rays and $^{12}C^{5+}$ ion beams, the 5-fluoroorotic acid-resistant mutant frequency was determined. The highest value of mutant frequency of $^{12}C^{5+}$ ion beams was obtained at 100 Gy, and it was over 10 times higher than that of gamma rays at 66 Gy. These highest values of mutant frequency were observed at a dose that resulted in a survival fraction of above 10% for both types of radiation [29].

The effects of different LET radiations on bacteria have also been investigated. The lethal effects of $^{12}C^{5+}$ ion beams on wet cells of *Escherichia coli*, a model gram-negative bacterium, were compared with those of gamma rays. The *E. coli* cells were much more sensitive to the $^{12}C^{5+}$ ion beams than to gamma rays (RBE value 1.8; Figure 2) [25]. The lethal effects of $^{12}C^{5+}$ ion beams on freeze-dried spores of *Bacillus subtilis*, a model gram-positive bacterium, were also compared with those of gamma rays. The result was the same as for *E. coli*; the $^{12}C^{5+}$ ion beams were much more effective (RBE value 2.9; Figure 2) [26]. Freeze-dried cells of *Rhodococcus erythropolis*, an aerobic gram-positive member of the phylum Actinobacteria, were also more sensitive to $^{12}C^{5+}$ ion beams than gamma rays (RBE = 2.1). Rifampicin is an antibiotic that inhibits RNA synthesis by directly interacting with the RNA polymerase beta subunit protein encoded by the *rpoB* gene. By disrupting the *rpoB* gene in the bacterial genome, rifampicin resistant mutants can be generated. The frequency of rifampicin resistant mutants can be used as an index to evaluate mutagenic effects on bacteria. In *R. erythropolis*, the frequency of rifampicin resistant mutants increased depending on the radiation dose. The highest mutant frequency for $^{12}C^{5+}$ ion beams was observed at a dose giving a surviving fraction of around 1% (Table 2) [30]. The effects of different LET radiations ($^{4}He^{2+}$, $^{12}C^{5+}$, $^{12}C^{6+}$, $^{20}Ne^{8+}$, and $^{40}Ar^{13+}$ ion beams and gamma rays) on another gram-positive bacterium, *Deinococcus radiodurans*, which is known as a radioresistant bacterium, were also investigated. The degree of radioresistance of freeze-dried *D. radiodurans* cells onto the cellulose membranes irradiated by all ion beams except $^{40}Ar^{13+}$ was almost equal to that to gamma rays. Ar ion-irradiated cells exhibited a much higher resistance than to the other radiations even at high doses (10 to 15 kGy). This higher resistance to Ar ion beams was attributed to an overkill effect, in which a much higher ionizing density is deposited on the target than the energy required to inactivate the cell. The rifampicin-resistant-mutant frequencies increased depending on the radiation dose regardless of the type of irradiation [27]. The highest mutant frequencies for all radiations were observed at a dose around D_{10} (Table 2) [33].

The series of experiments for *B. subtilis*, *R. erythropolis*, and *D. radiodurans* indicated that freeze-dried cells exhibit a higher resistance to gamma-irradiation than wet cells [27,30,34]. These results suggested that the effect of water radiolysis, which caused an indirect action of ionizing radiation, was lower in dry conditions, and that dry conditions could potentially alleviate the generation of mutations in the genome. Since the characteristic biological effects of high-LET radiations are thought to be attributable to direct action rather than indirect action of radiation, use of ion beams in dry conditions may lead to different induced mutations compared with gamma rays. However, since the viability of microorganisms decreases in dry conditions, it is harder to obtain high numbers of colonies after irradiation, and thus it is important to maintain high viability of the microorganisms in dry conditions by adding protective compounds such as skim milk, sodium glutamate, glycerol, or trehalose.

In summary, the RBE in many microorganisms depends on the LET and exhibits a peak in the LET range from about 100 to 400 keV/μm (Figure 2). Exceptionally, no RBE peak is observed for

D. radiodurans because the resistance of this bacterium to ion beams and gamma radiation is comparable. The dose range that gives a surviving fraction of 1% to 10% is effective for inducing mutations with high frequency. By using $^{12}C^{5+}$ ion beams in this dose range, a wide range of microorganisms have been improved successfully, suggesting that ion beams can be applied to breeding of useful microorganisms for the industry.

3. Ion-Beam Breeding of Microorganisms

The ion-beam breeding technology at TIARA has been applied to the development of >20 industrial microorganisms, including fungi, microalgae, and bacteria (Table 4). Some examples of applications of ion-beam breeding for improving useful microorganisms are presented below.

Table 4. Breeding of microorganisms using ion-beam breeding technology at the Takasaki Ion Accelerators for Advanced Radiation Application (TIARA).

Microorganism	Ion	Dose Range (Gy)	Breeding Objective	References
Fungi and Yeast				
Aspergillus awamori	$^{12}C^{5+}$	100 to 1200	High amylase activity	[35]
Aspergillus sojae	$^{12}C^{5+}$	100 to 500	High producing of protease	[36]
Aspergillus usamii	$^{12}C^{5+}$	10 to 1000	High decompose ability of dark brown pigments	[37]
Coriolus versicolor	$^{12}C^{5+}$	300	High decompose ability of dark brown pigments	[37]
Rhizomucor miehei	$^{4}He^{2+}$ $^{12}C^{5+}$ $^{20}Ne^{8+}$ $^{40}Ar^{13}$	100, 200	Low lipase activity Low coagulation activity	[31,32]
Beauveria bassiana	$^{12}C^{5+}$	50 to 400	High fungicide resistance	[38]
Isaria fumosorosea	$^{12}C^{5+}$	50 to 600	High fungicide resistance	[39]
Metarhizium anisopliae	$^{12}C^{5+}$	100 to 500	High thermotolerance	[40]
Pleurotus osutreatus	$^{12}C^{6+}$	50 to 1000	New variety	[41]
Lyophyllum decastes	$^{12}C^{6+}$	50 to 1000	New variety	[41]
Pleurotus eryngii	$^{12}C^{6+}$	50 to 1000	New variety	[41]
Ganoderma lucidum	$^{12}C^{6+}$	50 to 1000	New variety	[41]
Grifora flondosa	$^{12}C^{6+}$	50 to 1000	New variety	[41]
Alcohol fermentative yeast	$^{12}C^{5+}$	10 to 300	High producing of ethanol	[42]
Saccharomyces cerevisiae	$^{12}C^{5+}$	50 to 300	High producing of ethyl caproate	[43]
Zygosaccharomyces rouxii	$^{12}C^{5+}$	50 to 300	Auxotrophy	[44]
Microalgae				
Chlamydomonas sp.	$^{12}C^{5+}$	50 to 100	High salinity tolerance	[45]
Tisochrysis lutea	$^{12}C^{6+}$	5 to 320	High oil productivity	[46]
Bacteria				
Bradyrhizobium japonicum	$^{12}C^{5}$ $^{4}He^{2+}$ $^{12}C^{6+}$	50 to 800	High thermotolerance	[47]
Deinococcus radiodurans	$^{12}C^{5+}$ $^{20}Ne^{8+}$ $^{40}Ar^{13}$	2000 to 20000	Cs-accumulating ability	[48]
Pseudomonas fluorescens	$^{12}C^{5+}$	10 to 300	High suppression effect of tomato bacterial wilt	[49]
Rhodococcus erythropolis	$^{12}C^{5+}$	200 to 2000	Cs-accumulating ability	[30]
Streptomyces coelicolor	$^{12}C^{5+}$	10 to 1000	Pigment producing ability	[50]

3.1. Sake Brewing

Sake is a traditional liquor made from rice, but sake consumption has been decreasing year by year in Japan. To elevate sake consumption, it is important to quickly generate new varieties of sake, meeting the needs of the market. In addition to alcohol fermentation, sake yeast has a key role in producing its own flavor and taste characteristics. Ethyl caproate is an important flavor component in

Ginjo-shu (sake brewed with highly polished rice). Therefore, the breeding of sake yeast (*S. cerevisiae*) for high production of ethyl caproate has been attempted by using ion-beam breeding technology. Freeze-dried sake yeasts onto the cellulose membranes were irradiated with $^{12}C^{5+}$ ion beams in the dose range 50 to 300 Gy (Table 4). The most effective dose was 125 Gy. Following irradiation, over 2000 cerulenin-resistant sake yeast mutants were isolated. Ethyl caproate is biosynthesized from caproic acid, a medium-chain fatty acid, and ethanol. Cerulenin inhibits long-chain fatty acid synthesis. It was therefore expected that the productivity of medium-chain fatty acids including caproic acid (and hence ethyl caproate) would be different in cerulenin-resistant mutants from that in the parental strain. A high-ethyl-caproate-producing strain was selected from among the mutants. The production of ethyl caproate in the novel strain of sake yeast (7.4 ppm) was 4.6 times that in the parental strain (1.6 ppm; Figure 4). The novel yeast was shown to have the potential for commercial brewing by test brewing for three years [43]; Ginjo-shu prepared using this novel stain of sake yeast has been commercially available since April 2013. To find factors contributing to the high productivity of ethyl caproate in the novel yeast, DNA sequences of fatty acid synthase genes (*FAS1* and *FAS2*) and fatty acid esterase genes (*EHT1* and *EEB1*) that are involved in ethyl caproate production have been analyzed. No mutation was found in the *EHT1*, *EEB1*, and *FAS1* loci compared with the parental strain. However, the novel sake yeast strain carried a mutation (G to A) at nucleotide position 3748 in the *FAS2* gene. This mutation causes an amino acid substitution at position 1250 (Gly to Ser, G1250S) in the FAS2 protein [51]. Recombinant sake yeast carrying the same mutation in FAS2 exhibits a high ethyl caproate production phenotype [52], suggesting that the higher production of ethyl caproate in the sake yeast generated by ion-beam breeding is attributable to this dominant mutation in the *FAS2* gene.

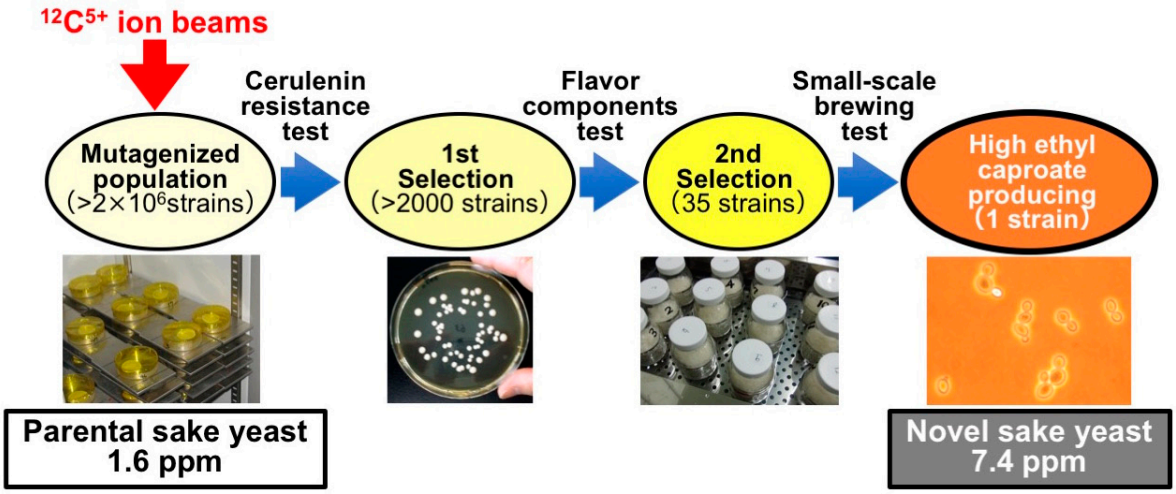

Figure 4. Development of high-ethyl caproate-producing sake yeast (*Saccharomyces cerevisiae*) by ion-beam irradiation. As the first selection, >2000 cerulenin-resistant mutants were isolated from a mutagenized population of sake yeast, which was generated by carbon-ion-beam irradiation. Next, 35 mutants were selected based on the amount of flavor components produced, including ethyl caproate. Finally, a high-ethyl caproate-producing sake yeast was identified by small-scale brewing tests. The novel sake yeast strain obtained exhibited over four times the ethyl caproate producing ability of the parental strain.

3.2. Industrial Enzyme Productions

Proteases and peptidases are very important enzymes in soy sauce production. These enzymes hydrolyze soybean and wheat proteins to peptides and amino acids, which form the basis for the taste of soy sauce. Therefore, mutants with high ability to produce proteases and/or peptidases are desired by the soy sauce industry. However, even if high-enzyme-producing mutants are acquired by conventional mutation methods, most such mutants have simultaneous undesirable characteristics, such as slow growth rate and decreased activity of other important enzymes, for example peptidase

and glutaminase. Therefore, ion beams were considered as alternative mutagens and wet conidia of *A. sojae* onto the agar plates were irradiated with $^{12}C^{5+}$ ion beams in the dose range of 100 to 500 Gy (Table 4). The most effective dose to induce mutant was 250 Gy. Following irradiation, high-protease-producing mutants were screened by observing the size of the halo around colonies on casein plates, and six high-protease-producing mutants were obtained. The protease activities of the mutants (about 1100–1930 U/g strain) were over two times higher than that of the parental strain (500–650 U/g strain) [36]. In this case, 1 unit (U) of the protease activity is showed as the amount that generates 1 µmol of tyrosine in 1 minute per 1g of *A. sojae*. While, productions of the peptidase and glutaminase were comparable in the mutant and parental strains. It was found in test brewing that the mutants could make a very strong tasting soy sauce, implying that they have the potential for practical use in soy sauce production [36].

Amylase is a very important enzyme that digests starch. For the effective use of starchy crops such as wheat, corn, potatoes, and cassava, it is important to digest raw starches for the industrial fermentation process. *Aspergillus awamori* is a fungus used to produce an acidophilic amylase. The high amylase activity mutants could not be acquired by a conventional breeding method by UV. Therefore, for increasing of amylase activity, freeze-dried spores of *A. awamori* onto the membranes were irradiated $^{12}C^{5+}$ ion beams in the dose range of 100 to 1200 Gy. The most effective dose to induce a mutant was 750 Gy. Following irradiation, the black colored and large size colonies were selected, tested their amylase activity individually, and three high amylase activity mutants were obtained. The amylase activities of the mutants (about 3500–5800 IU/L) were 1.4 to 2.4 times higher than that of the parental strain (2400 IU/L) [35]. In this case, IU means the amount of enzyme releasing reducing sugar equivalent to 1 µmol of glucose per one minute. Moreover, the frequency to induce useful mutant strains with high amylase activity was five to 10 times higher in $^{12}C^{5+}$ ion-beams irradiated samples than in gamma-irradiated ones [35].

3.3. Biopesticides

In addition to microorganisms for the use in the fermentation industry, ion-beam breeding technology has been applied to develop microorganisms for the use in agriculture and environmental technologies. The entomopathogenic fungi *Isaria fumosorosea*, *Beauveria bassiana*, and *Metarhizium anisopliae* are important agents for the biological control of insect pests and are commercially available as biopesticides. However, these fungi are highly susceptible to chemical fungicides used to protect plants from diseases such as powdery mildew. Thus, if biopesticides are used together with chemical fungicides, the biological control potential of the biopesticide is reduced. In addition, these fungi are susceptible to high temperatures. To overcome these problems, ion-beam breeding technology was applied to generate fungicide-resistant or thermotolerant mutants. Dried conidia of *I. fumosorosea*, *B. bassiana*, and *M. anisopliae* onto the cellulose membranes were irradiated with $^{12}C^{5+}$ ion beams in the dose range of 50 to 600 Gy (Table 4). The most effective doses to induce mutants of *I. fumosorosea*, *B. bassiana*, and *M. anisopliae* were 300, 150, and 300 Gy, respectively. For fungicide resistance, the mutants were screened in an inhibitory concentration of benomyl, a major fungicide. The obtained benomyl-resistant mutants of *I. fumosorosea* (survived in benomyl concentrations >5000 mg/L) and *B. bassiana* (>500 mg/L) were over 2000 and 500 times more resistant than the parental strains (2.5 and 1 mg/L, respectively) [38,39]. To select thermotolerant mutants, the irradiated conidia were cultivated at a high temperature (38 ± 1 °C), which was high enough to prevent the growth of the parental strains. Thermotolerant mutants of *M. anisopliae* showed an upper limit for vegetative growth of 38–39 °C, two to three degrees higher than the parental strain (36 °C) [40]. These useful mutants of entomopathogenic fungi were obtained without reducing their biological control potential, and expected to be used as new biopesticides.

3.4. Biofertilizers

Bradyrhizobium japonicum is a rhizobium that has the ability to elicit the formation of special organs called nodules on leguminous host roots for symbiotic nitrogen fixation. It is used in biofertilizer inoculants that promote plant growth. The development of biofertilizers has been progressed in Asian countries to increase crop yield under low input of chemical fertilizers. However, there is a problem with the use of *B. japonicum* biofertilizers, especially in south Asian countries, because they easily lose viability at a high temperature and under drought stress, including during storage and transportation. The thermotolerant mutant is desirable. For this purpose, freeze-dried *B. japonicum* cells onto the cellulose membranes were irradiated with $^{12}C^{5+}$ ion beams in the dose range of 50 to 800 Gy (Table 4). The thermotolerant mutant was obtained from cells irradiated at 300 Gy. The mutant could survive at 42 °C, at which growth of the parental strain is prevented, without affecting the nodulation and nitrogen fixation abilities [47]. This thermotolerant mutant is expected to be used as a new biofertilizer inoculant. To clarify factors contributing to the high thermotolerance, the whole genome sequence of the mutant has been analyzed. Although detailed analyses of the mutated genes are still in progress, genome comparison analysis revealed that a large-scale structural change (an inversion of 1.27 Mb) and 18 single base substitutions occurred in the 9.11-Mb chromosome of the mutant [53].

3.5. Bioremediation

Microorganisms have often been used to develop bioremediation technologies to remove and recover contaminated environments. Since the Fukushima Daiichi nuclear disaster, removing radioactive caesium from contaminated soil has been one of the most important issues in Japan. In this context we have worked on bacterial strains, *R. erythropolis* CS98 that was isolated from soil as a radioactive Cs-accumulating bacterium [54], and *D. radiodurans*, which also possesses a Cs-accumulating ability [55], to investigate mechanisms of Cs accumulation in these bacteria. To generate mutants with altered Cs-accumulating ability, freeze-dried cells of *R. erythropolis* CS98 and *D. radiodurans* onto the cellulose membranes were irradiated with $^{12}C^{5+}$ ion beams in the dose ranges of 200 to 2000 and 2000 to 20000 Gy, respectively (Table 4). The effective dose of *R. erythropolis* CS98 and *D. radiodurans* were 800 and 8000 Gy, respectively, that resulted in a surviving fraction of 1% to 10%. Mutants with an altered Cs-accumulating ability were screened by measuring intracellular Cs levels using an atomic absorption spectrometer. Consequently, candidates of low Cs-accumulating mutants of *R. erythropolis* CS98 [30] and high Cs-accumulating mutants of *D. radiodurans* were obtained [48]. Genome analyses of the mutants are still in progress and will clarify the factors involved in Cs-accumulation.

3.6. Biofuels

Biofuels, including bioethanol and biological oils, which are produced through metabolic processes in yeasts or microalgae, have recently attracted significant attention as candidate renewable energy sources. Highly efficient production of biofuels from non-edible biomass resources, such as starch and lignocellulose, is a very important issue for the biomass industry. During bioethanol production in yeast, ethanol production is negatively affected when cells are exposed to a high temperature and toxic compounds derived from chemical pretreatment processes or hydrolyzation for saccharification and fermentation of the substrate [56]. Therefore, the development of mutants that are tolerant to a high temperature and toxic compounds is desirable. To obtain such mutants, wet cells of alcohol fermentative yeast onto agar plates were irradiated with $^{12}C^{5+}$ ion beams in the dose range of 10 to 300 Gy (Table 4). For inducing a mutant, the dose at 200 Gy was the most effective. A high-ethanol-producing mutant was obtained by screening at a high temperature (35 °C) in medium containing bagasse sugar lysate as a toxic compound [42]. The ethanol producing ability of the mutant (about 20 g/L) was over three times that by the parental strain (about 6 g/L) [57].

Microalgae are promising biofuel producers because their lipid productivity per unit area is higher than that of land plants, and sea water can be used for mass culture [58]. The salinity tolerance and high

lipid productivity are important factors. To improve the salinity tolerance and lipid productivity of a typical microalga, wet cells of the *Chlamydomonas* sp. onto the agar plates were irradiated with $^{12}C^{5+}$ ion beams at 50 or 100 Gy (Table 4). As a result, high-salinity-tolerant mutants with an ability to grow in 7% sea salt were obtained [45]. The salinity-tolerant mutants exhibited high biomass production (about 4.1 g/L) in the high salt conditions, in which the parental strain could hardly produce biomass (about 0.3 g/L) [45].

The results described above suggest that ion beams can be used as powerful mutagens and are effective for breeding industrial microorganisms. Therefore, it could greatly contribute to various fields, such as securing stable food production, agriculture, environmental conservation, and the development of bioenergy resources, using ion-beam breeding technology.

4. Ion-Beam Breeding of Microorganisms in Other Facilities

In addition to TIARA at QST, irradiation facilities that can be used for ion-beam breeding technology in Japan are the RI Beam Factory at RIKEN (RIBF) [59], the Multi-purpose Accelerator System with Synchrotron and Tandem at the Wakasa Wan Energy Research Center (W-MAST) [60], and the Heavy Ion Medical Accelerator in Chiba (HIMAC) at the National Institute of Radiological Sciences, QST [61]. In China, ion beams are applied for improving microorganisms at the Heavy Ion Research Facility in Lanzhou (HIRFL), the Institute of Modern Physics, Chinese Academy of Sciences [62]. Other Asian countries are also paying attention to the use of ion beams for mutation breeding. Recently, the ion-beam breeding technology at RIBF, RIKEN has been applied to generate high-biofuels-producing mutants of microalgae species such as *Parachlorella kessleri* and *Euglena gracilis* [63,64]. At W-MAST, the high cordycepin producing mutants of the medicinal mushroom *Cordyceps militaris* have been obtained successfully [65]. At HIMAC, the ion beams have been used to clarify the resistance of *B. subtilis* spores to ionizing radiations [66]. More than 20 kinds of useful microorganisms, including bacteria, fungi, and microalgae, have been bred with the ion beams provided by HIRFL [62]. The ion beams have been demonstrated as the effective novel mutagen for breeding technology at these irradiation facilities. These results have attracted much attention in both the industrial and academic fields. We expect that the accessibility of this technology will be ensured in the future by establishing effective utilization systems in the available irradiation facilities, and support systems for users. Ion-beam mutagenic technology in microorganisms would contribute to better human life.

5. Conclusions

In this review, the application of ion-beam mutation technology at TIARA for breeding of various microorganisms, such as fungi, algae, and bacteria was described. Mutation breeding is a very important technology to expand the available variety of biological resources. As in the case of higher plants, ion beams cause highly lethal and mutagenic effects, a low multitude of mutations in a locus, and large-scale genomic variations such as large deletions, translocations, and inversions, in microorganisms. Studies described in this review suggest that the application of ion-beam mutagenic technology to microorganisms is useful in both basic science and applied research.

Although it is very important to select the LET of ion beams for effectively inducing a mutation, the differences of mutagenic effects from the different LET of ion beams are poorly understood in a microorganism. In microorganisms, the RBE depended on the LET and the highest RBE values were obtained with $^{12}C^{5+}$ ion beams (107 keV/μm; Figure 2). LET represents the energy deposition of ionizing radiations on the target. The high density of deposited energy can cause more severe DNA damage. As the large-scale variations can affect the function of many genes, it is expected to obtain the mutants effectively. In tabaco single cells, initial yields of DNA double-strand breaks (DSBs) by the different LET (94.8 to 431 keV/μm) of carbon ion beams depended on LET as well as the RBE based on the lethality, and the highest DSBs yields were obtained at 124 and 241 keV/μm [67]. In *A. thaliana*, it has been shown that carbon ions near the range end (425 keV/μm) frequently cause

large-scale deletions compared with carbon ions penetrating the seeds (113 keV/μm) [68]. Moreover, in rice, the mutation frequency by the two different LET carbon ion beams (76 and 107 keV/μm) has been investigated [69]. The frequency of high LET carbon ion beams was higher than that of the low LET carbon ion beams. These indicate that the LET of ion beams is an important factor for biological effects in a plant. The $^{12}C^{5+}$ ion beams in anticipation of high mutagenic effects have been usually used for the ion-beam breeding of a microorganism at TIARA. As a result, many mutants have been obtained successfully.

For irradiation to biological samples, at RIBF, five kinds of ion beams ($^{12}C^{6+}$ (23 keV/μm), $^{14}N^{7+}$ (61 keV/μm), $^{20}Ne^{10+}$ (62 keV/μm), $^{40}Ar^{17+}$ (280 keV/μm), and $^{56}Fe^{24+}$ (624 keV/μm)) are used as typical ones. At W-MAST, $^{1}H^{+}$ (0.45 keV/μm) and $^{12}C^{6+}$ (56 keV/μm) are used. At HIMAC, five kinds of ion beams ($^{12}C^{6+}$ (13 keV/μm), $^{20}Ne^{10+}$ (30 keV/μm), $^{28}Si^{14+}$ (54 keV/μm), $^{40}Ar^{18+}$ (89 keV/μm), and $^{56}Fe^{26+}$ (185 keV/μm)) are used. At HIRFL, $^{12}C^{6+}$ ion beams (30 to 40 keV/μm) are used. The LET ranges of provided ion beams from these facilities differ from those of TIARA. The availability of many different LET ion beams is a great advantage for mutation-breeding technology. The radiosensitivity of microorganisms to ion beams varies extensively depending on the species. For the effective mutagenesis in a microorganism, the investigation of lethal effects for different LET radiations and the selection of suitable LET value at radiation doses that gave 1%–10% of the surviving fraction for the target microorganisms would be important. Moreover, the genome analyses of the obtained mutants will be helpful to clarify the useful characteristics. Recently, the genome comparison analysis of the thermotolerant *B. japonicum* mutant has been revealed the mutations including an inversion of 1.27 Mb and 18 single base substitutions in the 9.11-Mb chromosome [53]. The genome analyses of the other ion-beam-induced mutants are in progress to clear the differences of mutational functions induced by different LET radiations in microorganisms. Further characterizations of mutations induced by different LET radiations will facilitate a more effective use of ion beams in microorganisms breeding.

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