FLAVORATOR

Increased production of fragrance for new food preservation method by oder *E.coli*

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Abstract

We thought about how to solve the food shortage problem, last year.We considered method to save the food without using electricity.We devised Flavorator which preserve food by fragrance component with antibacterial effect and introduced its concept. We chose geraniol and farnesol as the fragrance having an antibacterial effect, and we made E.coli possible produce these fragrance components by recombination. However, the synthetic amount of geraniol and farnesol was lower than expected, and it is not sufficient for practical use. To increase farnesol production, we improved farnesol synthetic process by adding three genes to E.coli from the last year. 1, The gene that increases the intermediate product of farnesol synthesis. As 1-deoxy-D-xylulose 5-phosphate (DXP), a key gene for farnesol synthesis, is also used for another compound synthesis, we added a new DXP synthase to increase the intermediate product. 2, The dephosphorylation enzyme gene Last year, a synthesis of farnesol relied on endogenous dephosphorylation enzyme of E. coli, and this could be a bottleneck for farnesol production. This year, we introduced an additional endogenous phosphatase gene (YbjG, PgpB) in E. coli to increase the production of farnesol. 3, A gene to improve resistance against farnesol. As farnesol is an antibacterial fragrance, it is toxic to E.coli itself. So, we introduced the endogenous activator gene of AcrAB-TolC efflux pump, marA to improve resistance against farnesol Also, to improve the feasibility of Flavorator in the real situation, we thought that it is important to produce farnesol by non-recombinant E.coli. Therefore we attempted to increase production of farnesol using CRISPER / Cas9 system. E. coli has their pathway to produce the farnesol. However, the gdhA gene, which is not lethal to E.coli, produce a byproduct from farnesol synthetic pathway and decrease the production of farnesol. We tried to knockout gdhA, gene did not relate to falnesol synthesis, with CRISPER-CAS9 and be accumulate intermediate metabolite.

Project

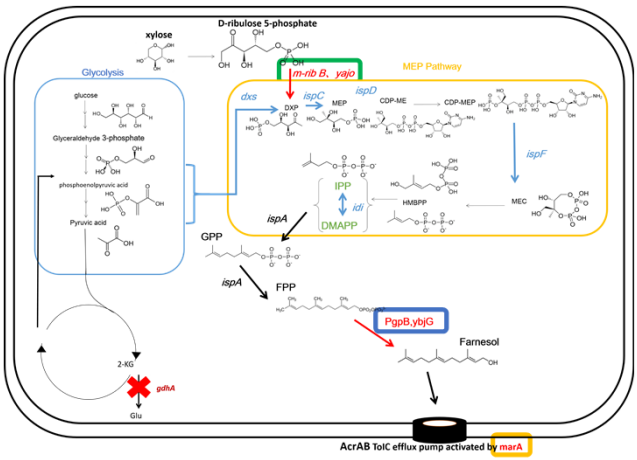


Fig.1 Pathway of our design We tried to knockout gdhA, gene did not relate to falnesol synthesis, with CRISPER-CAS9 and be accumulate intermediate metabolite. DXP: 1-Deoxy-D-xylulose 5-phosphate. MEP: 2-C-methylerythritol 4-phosphate. CDP-ME: 4-diphosphocytidyl-2-C-methylerythritol. CDP-MEP: 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate. MEC: 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate. HMBPP: (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate. IPP: Isopentenyl pyrophosphate. DMAPP: Dimethylallyl pyrophosphate. GPP: geranyl diphosphate. FPP: farnesyl diphosphate.

Result1~4
1.Confirmation antibacterial activity of farnesol
Result 5
2.Enhancement of farnesol resistance
Result 6
3.Confirming of amount of transcription of fusion gene
Result 7
4.Confirming of amount of transcription of ybjG and pgpB

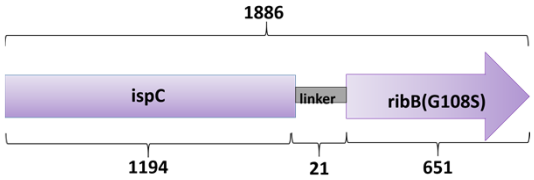


Fig.2: ispC is one of genes in MEP pathway. m-ribB（G108S）is a gene for synthesizing DXP from other pathways.Fusion gene is a gene that combines those genes.

Result

1. Comparing antibacterial of each antibacterial substances using bread

We examined our working hypothesis to “Flavorator” that farnesol can show either the antibacterial or bacteriostatic activity in a box like “KOZOKO”. The results clearly showed that farnesol had antibacterial properties.

Fig.3:Comparison of the suppressing effect of geraniol, farnesol and ethanol on mold
A: geraniol(100 µl of geraniol solution was dropped on the cotton）B:farnesol(100 µl) C: ethanol (100 µl) A:Chopstick dipped in suspension of mold was touched in the center of the bread. The volume of box is 846cm³. B:The same treatment was done. C:The same treatment was done. These boxes were kept for 5 days at room temperature.

2. Confirming antibacterial activity of farnesol using various food.

Farnesol has high antifungal activity against the mold of bread. Therefore, we investigated whether farnesol exerts similar antifungal effects on other food.We found that farnesol has a preservative effect on various foods.

Fig.4:Effects of farnesol on rice mold
A:farnesol(1ml of farnesol solution was dropped on the cotton), Chopstick dipped in suspension of mold was touched in the center of the rice.B:ddH2O(1ml). : The same treatment was done. These boxes were kept for 8 days at room temperature.

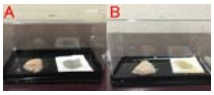


Fig.5:Effects of farnesol on maggot.
A:farnesol(1ml of farnesol solution was dropped on the cotton)Chopstick dipped in suspension of rotten meat was touched in the center of the chicken.B:ddH2O(1ml): The same treatment was done.These boxes were kept for 8 days at room temperature.

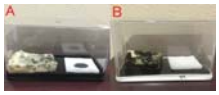


Fig.6:Effects of farnesol on bread mold
A:farnesol(1ml of farnesol solution was dropped on the cotton), Chopstick dipped in suspension of mold was touched in the center of the bread.B:ddH2O(1ml)The same treatment was done. These boxes were kept for 8 days at room temperature.

3. Effect of farnesol on growth of *E.coli*

This result indicates that farnesol affect *E.coli.* , so we hypothesized that *E.coli* needs to have resistance to farnesol.

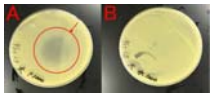


Fig.7:Effect of farnesol on growth of *E.coli*
A: farnesol (1ml) B: ddH2O (1ml) Farnesol was dropped on the paper which was put on center of the plate cover without direct contact with the bacteria. These plates were incubated for 21 hours at 37 ℃. Growth inhibition circle was not observed on the plate(B). While, inhibition circle was observed on the plate with farnesol (A).

4. Effect of farnesol on the growth Staphylococcus SP. like

We examined whether farnesol also has an effect against food poisoning bacteria. We examined fungus that presumed Staphylococcus aureus from the human palm and we isolated it.We examined whether farnesol inhibits the growth of it.

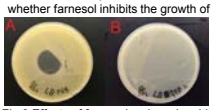
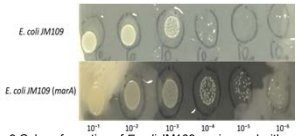


Fig.8:Effects of farnesol on bread mold
A: farnesol(1ml of farnesol solution was dropped on the cotton), B:ddH2O(1ml). A:Chopstick dipped in suspension of mold was touched in the center of the bread. The volume of box is 846cm³. B: The same treatment was done. These boxes were kept for 8 days at room temperature

5. Enhancement of farnesol resistance

We introduced an activator gene of AcrAB-TolC efflux pump (marA) to release the farnesol from the cells. In our study, we confirmed that overexpression of marA improve resistance against farnesol.



E.coli JM109 and *E.coli* JM109 (marA) were dropped on LBGMg agar plates in serial ten-fold dilutions (10⁻¹～〖10〗^(-6)), overlaid with 30 % (v/v) farnesol hexane solution (farnesol solution) and incubated at 30° C for 24 h. This figure shows that *E.coli* JM109 (marA) cells that overexpress the marA product better than the control *E.coli* JM109 wild type cells survived plates overloyed by 30 % farnesol solution.

Fig.9:Colony formation of *E.coli* JM109 engineered with marA on farnesol overlaid plates.

6. Confirmation of Amount of transcription of fusion gene

From this result, the transcription of mribB and ispC is being performed, it can be said that the construct of the fusion protein was successful

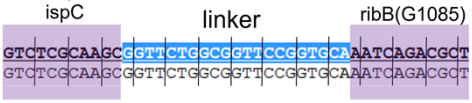


Fig.10:Linker sequence between ispC and ribBG108S
Above sequence is reference data of ispC-ribBG108S, and blue colored sequence is linker G2. Blow sequence is this part data. Two sequences are matched. This proved that ispC and ribBG108S are linked by linker G2.

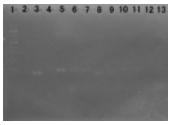


Fig.11:The result of RT-PCR fusion gene
Lane1:100bp DNA ladder
Lane2:ispC(WT)
Lane3:ispC(recombinant)
Lane4:m-ribB(WT)
Lane5:m-ribB(recombinant)
Lane6:gapA(WT)
Lane7:gapA(recombinant)
Lane8:ispC(WT) Negative control
Lane9:ispC(recombinant) Negative control
Lane10:m-ribB(WT) Negative control
Lane11:m-ribB(recombinant)Negative control
Lane12:gapA(WT) Negative control
Lane13:gapA(recombinant)Negative control

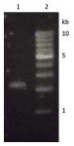


Fig.12:Gel electrophoresis of colony PCR products

Rane 1 is BBa_K1950010. Rane 2 is 1 kb step DNA Ladder (Promega). BBa_1950010 is amplified by VF2 primer and VR primer. PCR product length expected 2254bp

7. Confirmation of Amount of transcription of ybjG and pgpB

We thought that gDNA could be removed ,because band was not appeared any lane. Therefore, we thought that the amount of transcription of pgpB was increased.

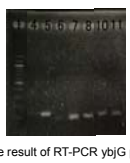


Fig.13The result of RT-PCR ybjG pgpB

Lane1:100bp DNA ladder,
Lane4:ybjG (WT),
Lane5:ybjG (ybjG),
Lane6:pgpB (WT)
Lane7:pgpB (pgpB),
Lane8:gapA(WT),
Lane10:gapA (ybjG),
Lane11:gapA (pgpB)

Future work

1. We transform finished fusion gene in E. coli, which synthesizes farnesol.As a result, farnesol will be increased.

2. We will finish knockout of extra intermediate metabolite synthesis gene,Unnecessary gene for farnesol synthesis, using CRISPR / Cas9.And it will lead to the realization of farnesol synthesis of non-recombinant E.coli

Achievement

Bronze Medal
- [x] We are joining Giant Jamboree.
- [x] We achieved all requirements page (section 3).
- [x] We created clear attribution of each aspect of our project.
- [x] We created and submitted 3 new BioBricks for bronze medal criterion.

Silver Medal
- [x] We validated that ispC-ribB(G108S) fusion experimentally.
- [x] We collaborated with other iGEM teams.
- [x] We informed our project and synthetic biology to the local and Japan.

Gold Medal
- [x] We integrated many ideas given from general public.
- [x] We did characterization of an existing BBa K1230000（MarA）.
- [x] We demonstrated a functional proof of concept of our project by confirmation of farnesol resistance by MarA.
- [x] We demonstrated FLAVORATOR working under real-world conditions.

Human Practice

▪Nagahama Rotary Club

"Nagahama Rotary Club" is Rotary Club that is active in Nagahama.

Rotary Club is an international community service federation.The most important activities of Rotary Club is to enhances its professional members’ ethics and to contribute to society.
Leaders in variety occupations or are Rotary club members participate in Rotary Club actuation.We have explained synthetic biology and our project to the leaders of various occupations in Nagahama.

▪TV show

Nippon Hoso Kyokai Osaka broadcasting station
“School re-discovery Variety AHOYANEN SUKIYANEN”

This program was a program for students. It was broadcasted in the Kansai area. We had a chance to appearance on TV and to spread our project and synthetic biology. We discussed our project, synthetic biology, safety of genetically modified foods with the cast. It was very valuable time for us to have a discussion with them.