Aspergillus oryzae is good at usage of hydrolyzing enzyme on solid-surfaces
— Recruitment of polyesterase (cutinase) of A. oryzae by the biosurfactant protein hydrophobin RolA on plastics

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TOPICS

• *Aspergillus oryzae* and Biodegradable Plastic Recycle System
  - Process design
  - Plastic-degrading enzyme CutL1 and a novel plastic-degrading factor hydrophobin RolA

• Novel Molecular Functions of RolA
  - Recruitment of soluble esterase CutL1 by RolA attached to hydrophobic surfaces
Fungi degrade biopolymers in nature

1. Infection

2. Hyphae
   - Surface active proteins
   - Hydrolysis of polymers
   - Penetration

3. Degradation enzymes for biopolymers

4. Pathogens
PBSA mimics wax polyesters

X. Fang, et al., NMR studies of molecular structure in fruit cuticle polyesters, *Phytochemistry*, 57 (2001) 1035-1042

- **Surface materials of plants**
- **Wax Polyesters** (**= biodegradable**)
Monomer Recycle System of Biodegradable Plastics by *A. oryzae*
PBSA recycling system mimics koji-fermentation

Soy bean

Wheat

A. oryzae

NaCl solution

filter-press

Soy sauce

koji

Moromi mush

Plastic koji

Plastic moromi

Extraction

PBSA monomers

1,4 butandiol

Succinate

Recycling
PBSA degradation by *A. oryzae* Rl B40

PBSA; polybutylene succinate co-adipate

degradation by cutinase (CutL1)
(Maeda *et al*. 2005; AMB)

1,4 - butanediol  succinate

PBSA-CD Agar Plate

PBSA-CD Liquid Medium
CutL1, PBSA degrading esterase produced by *A. oryzae*

**SDS-PAGE analysis of purified CutL1**

**Purified CutL1**

**Filter paper**

**PBSA film**

Incubation at 37 °C for 6 hours

Removal of the filter paper

**CutL1**

- 

+ 

**PBSA film**

The schematic model of PBSA-degradation by CutL1

Degradation of a PBSA film by CutL1

What is hydrophobin?

- **Feature**
  - conserved 8 cysteine residues
  - surface active

- **Localization**
  - aerial hyphae
  - outer - Hydrophobic phase
  - no expression in liquid culture

- **Function**
  - protection layer
  - resistant to drying

**Hydrophilic** and **Hydrophobic**

**Biosurfactant**
Alignment of deduced amino acid sequences of RolA homologs

<table>
<thead>
<tr>
<th>Aspergillus oryzae RolA</th>
<th>1</th>
<th>M•QFS- VAVLALATAVAALPPA- SGTGAGQVGH- SK- N•DFPLPKELT</th>
<th>44</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus fumigatus Hyp1</td>
<td>1</td>
<td>M•KFSLSAAVLAFASVAAL- PQHDVNAAGNGVGNKGNANVFVPDPDI T</td>
<td>48</td>
</tr>
<tr>
<td>Aspergillus nidulans RodA</td>
<td>1</td>
<td>M•KFSI AAANVFAAASVAALPPAHDASQFAGNGVGNKGNANVKFVPVENV T</td>
<td>49</td>
</tr>
<tr>
<td>Lentinula edodes Hyd2</td>
<td>1</td>
<td>M•QF- --KL- AF- VSI- AL- --A- TLA- --VAT- P- APRGE</td>
<td>25</td>
</tr>
<tr>
<td>Flammulina velutipes Fvh1</td>
<td>1</td>
<td>MVSF- -- R- -AFTVA- ASL- --FA- TLA- --AAT- P- LDTAL</td>
<td>27</td>
</tr>
</tbody>
</table>

| Aspergillus oryzae RolA       | 45 | TKQADDKGDQAOQLT- SGNKTVKTGFTQVEEGLXAGLLSNLLAGGQSOSG | 94 |
| Aspergillus fumigatus Hyp1    | 49 | VKQATEKCSDQAOQLS credits KATYAGDVTDI DEGI LAGTLKNLIGGS GTEG | 98 |
| Aspergillus nidulans RodA     | 50 | VKQASDKCDSDQAOQLS credits KATYAGDVTTVDEGLLSGALSGLIGAGSGAE | 99 |
| Lentinula edodes Hyd2         | 26 | P- -- ASS- C- STGDLQQCN- TVEPA- --SSPSASTILGLLIGIV- IQGVDVL- | 66 |
| Flammulina velutipes Fvh1     | 28 | PR- AADQC- NVSNQQQCN- SVQQA- --SSGPAAILGLLGTV- LQDVNL- | 70 |

| Aspergillus oryzae RolA       | 95 | LGLLQDOCTNI- PVI P- I- ISI AS- PQECKQPISCGQONTKSSADGDGLVGI | 140 |
| Aspergillus fumigatus Hyp1    | 99 | LGLFQCGSNVLQDI PVIGIPI QALVQNKCKQNI ACCQNSPSDASGLGILG | 148 |
| Aspergillus nidulans RodA     | 100| LGLFDQKAKLDVA- LI G- --I QDQ VQKCKQNI ACCQNSPSADGNLGIV | 146 |
| Lentinula edodes Hyd2         | 67 | VGL- --TC- --SP- I TV- I GLOSEGG- CSAQAVGC- --TDN- SNGGLISI | 102 |
| Flammulina velutipes Fvh1     | 71 | VGL- --DC- --SP- I TV- I GGGNGG- CNASPVCC- --ENN- SFGLISI | 106 |

<p>| Aspergillus oryzae RolA       | 141| GLPCI ALGSL | 151|
| Aspergillus fumigatus Hyp1    | 149| GLPCIALGISL | 159|
| Aspergillus nidulans RodA     | 147| GLPCIVALGISL | 157|
| Lentinula edodes Hyd2         | 103| G- -- CLP- -- VTL | 109|
| Flammulina velutipes Fvh1     | 107| G- -- CVP- -- ISI | 113|</p>
<table>
<thead>
<tr>
<th>Strains</th>
<th>Degradation ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. oryzae</em> wt</td>
<td>7</td>
</tr>
<tr>
<td>RolA overexpressing strain</td>
<td>25</td>
</tr>
<tr>
<td>CutL1 overexpressing strain</td>
<td>61</td>
</tr>
<tr>
<td>CutL1, RolA co-overexpressing strain</td>
<td>71</td>
</tr>
</tbody>
</table>
• *Aspergillus oryzae* and Biodegradable Plastic Recycle System
  - Process design
  - Plastic-degrading enzyme CutL1 and a novel plastic-degrading factor hydrophobin RolA

• **Novel Molecular Functions of RolA**
  - Recruitment of soluble esterase CutL1 by RolA attached to hydrophobic surfaces
Purification of RolA from culture broth of *A. oryzae* overexpressing RolA

**RolA overexpressing A. oryzae conidiaspore suspension**
- inoculated (1x10^6 spore per ml) to CD medium (1 % Fructose)
- cultured at 30°C for 48 hr
- filtrated with MIRACLOTH

**Culture broth; A**
- added solid (NH₄)₂SO₄ to 40 % saturation; B
- centrifuged 12000 g, 4°C, 20 min

**Supernatant**
- Octyl-Cellulofine type S column; C
- S-Sepharose FF column; D

**Purified RolA**
Effect of RolA on PBSA degradation by CutL1

- With pre-adsorption of RolA
  
  purified RolA + PBSA microparticle → 30°C, 16h adsorption → purified RolA + purified CutL1 → 40°C, 0-3h degradation

- Without pre-adsorption of RolA
  
  purified RolA + purified CutL1 + PBSA microparticle → 40°C, 0-3h degradation

Takahashi et al. Mol. Microbiol. 2005
Adsorption of CutL1 onto a PBSA film pre-coated with RolA

A. Immunostaining (anti-RolA antibody)
   + purified CutL1

B. Incubate at 37° for 6 h (PBSA degradation)

C. Immunostaining (anti-CutL1 antibody)

Takahashi et al. Mol. Microbiol. 2005
A quartz crystal microbalance (QCM) is a sensitive mass-measuring device. Its resonance frequency decreases linearly upon the increase of the mass on the QCM electrode at nanogram levels. Change of 100Hz of frequency corresponds to 3ng of protein bound to the QCM electrode.
About QCM

**Electrode attached with RolA**

- Oscillation frequency down indicates **Molecular interaction**

- $K_D$ values which indicate binding affinity can be analyzed by monitoring oscillation frequency change.
RolA (ligand) - CutL1 (analyte) interaction

Concentrations in the figure indicate final concentrations of CutL1 and BSA in QCM step-wise analyses.

Takahashi et. al. Mol. Microbiol. 2005
CutL1 (ligand) - RolA (analyte) interaction

Curve C
CutL1 immobilized QCM electrode

Curve D

RolA
BSA (control)

※ Concentrations in the figure indicate final concentrations of RolA and BSA in QCM step-wise analyses.

Takahashi et. al. Mol. Microbiol. 2005
Preparation of CutL1 mutants for RolA-CutL1 binding assay

Six acidic amino acid residues (Glu$^{31},$ Glu$^{109},$ Asp$^{142},$ Asp$^{145},$ Asp$^{171},$ Asp$^{203}$) were substituted with Ser, and all variants produced by *A. oryzae* were purified.
RolA-CutL1 binding assay

Purified CutL1 and its variants

Incubated at 30°C for 1h

Spin-down

Teflon-RolA complex

SDS-PAGE

Free CutL1

[Diagram showing the binding assay process with pH values and gel electrophoresis results]
Acidic amino acid residues (Asp, Glu) of CutL1 involved in CutL1-RoIA interaction.

E31, D142, D171 are important residues for CutL1-RoIA interaction.
Schematic model of RolA-CutL1 interaction

Abe et al. unpublished results
Models of PBSA degradation by RolA with CutL1
SUMMARY

• RolA adsorbed to the PBSA surface recruits CutL1 to the surface, resulting in condensation of CutL1 and stimulation of PBSA hydrolysis.

• Glu31, Asp142 and Asp171 of CutL1 are critically required for the RolA-CutL1 interaction by multivalent effect.

• H32 and K34 of RolA are important for the RolA-CutL1 interaction.
ACKNOWLEDGEMENT

• RolA
  Muragaki K., Uehara K., Ohataki S., Maeda H, Yamagata Y., Gomi K., Hasegawa F. (Tohiku Univ.)

• A. oryzae genomics
  Machida M. and Asai K. (AIST)

• Recycle of PBSA
  Ishioka R. and Okino Y. (Showa High Polymer)

• CD analysis of RolA
  Wang X. and Robillard G.T. (Groningen Univ.)

• FRAP analysis
  Kato M., Komura M. and Sato M. (Olympus Corp.)

• QCM analysis
  Jitsukawa T. (Inisium Corp.)

• AFM analysis
  Hondo H. (Ritsumeikan UNIV)
REFERENCES


Fluorescence recovery after photo-bleach (FRAP)

A. pre-Bleach
- PBSA film
- FITC labeled RolA

B. Bleach
- Laser beam (high power)

C. post-Bleach
- Fluorescence recovery after photo-bleach (FRAP)

D. post-Bleach

Graph:
- Time on the x-axis
- Fluorescence intensity on the y-axis
- A: 100%
- B: 0%
- C: Recovery
- D: Post-bleach recovery
FRAP analysis of FITC-RolA adsorbed to PBSA films

FITC labeling of RolA
↓
Immobilization of FITC-RolA on PBSA film
↓
PBSA surface is overplayed with (A) buffer, (B) CutL1, (C) BSA, or (D) anti-RolA antibody
↓
FRAP analysis

- RolA is laterally mobile on PBSA.
- Proteins that can interact with RolA inhibits the mobility.

Takahashi et. al. Mol. Microbiol. 2005
A phylogenetic relationship between HsbA and 4MeS

The phylogenetic tree of HsbA and its orthologues

4MeS

- Produced by a entomopathogenic fungus
- Transcribed in the fungal cells growing on the insect cuticle
- Function unknown
Expression and purification of recombinant HsbA
Recruitment of CutL1 on HsbA adsorbed on the PBSA surface

1. HsbA immobilization

2. + -

3. CutL1

4. Detected by immunostaining using anti-HsbA or anti-CutL1 antibody
Interaction between soluble CutL1 and HsbA adsorbed on the hydrophobic surface by QCM analysis

Soluble HsbA did not interact with soluble CutL1. (immunoprecipitation)

Ohtaki et al., Appl. Environ. Microbiol. 2006