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Chapter 16.

Fusion reporter approaches to monitor transmembrane helix interactions in bacterial membranes

Running head: protein-protein interaction in the membrane

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Summary: In transenvelope multiprotein machines such as bacterial secretion systems, protein-protein interactions not only occur between soluble domains but are also mediated by helix-helix contacts in the inner membrane. Here, we describe genetic assays commonly used to test interactions between transmembrane α -helices in their native membrane environment. These assays are based on the reconstitution of dimeric regulators allowing the expression of reporter genes. We provide detailed protocols for the TOXCAT and GALLEX assays used to monitor homotypic and heterotypic transmembrane helix-helix interactions.

Key words: membrane protein, protein-protein interaction, transmembrane segment, helix-helix interaction, homotypic, heterotypic, one-hybrid, two-hybrid, cI repressor, TOXCAT, GALLEX, BACTH.

1. Introduction

The proper assembly of multiprotein complexes such as bacterial secretion systems requires specific interaction between the different subunits. While most of the interactions involve contacts between soluble domains of these subunits, the transmembrane helices (TMH) of inner membrane proteins are also key players in membrane protein complex formation. For examples, the Type II secretion (T2SS) associated GspC, GspL and GspM proteins interact with each other via their TMH (*1*). A similar situation has been evidenced for the Type VI secretion system (T6SS) TssLM complex (*2-4*). The TMH could be involved in homotypic interaction, *i.e.*, participate to the formation of dimers such as the Type IV secretion (T4SS) and T6SS-associated VirB10 and TssL inner membrane proteins (*4, 5*) or in heterotypic interactions with other subunits (*1-3*). Monitoring interactions within TMH is not an easy task, as mutations within or swapping of the TMH could interfere with the conformation of

the soluble domains, and therefore may indirectly affect protein-protein interactions. Genetic one- or two-hybrid approaches based on fusion to transcriptional reporters, such as the λ cI repressor, TOXCAT, GALLEX and BACTH assays have been thus developed. While cI-repressor and TOXCAT can only be used for testing homotypic interactions, the GALLEX and BACTH approaches can also be used to monitor interactions between different TMH. This chapter provides protocols to monitor homotypic and heterotypic transmembrane helix-helix interaction using TOXCAT and GALLEX. We refer the reader to excellent reviews summarizing the forces exerted to catalyse trans-membrane helix folding and insertion, as well as the different methods to analyze transmembrane helix interactions in bacteria (6, 7).

1.1. Monitoring TMH homotypic interactions.

Methods to test homodimerization of transmembrane helices are based on the one-hybrid reporter fusion approach.

λ cI repressor. The cI transcriptional regulator represses the expression of early promoters of the bacteriophage λ genome. Repression only occurs when cI dimerizes, a behaviour conferred by the C-terminal domain. The λ cI repressor assay is therefore based on the reconstitution of a dimeric λ cI repressor by two interacting fragments (8-10). The construct consists to a fusion between the monomeric N-terminal DNA-binding domain of λ cI (called cI') with the TMH (Figure 1A). TMH-mediated cI' dimerization induces binding of cI to its operator sequence allowing repression of phage λ early genes, hence conferring protection against superinfection by phage λ (Figure 1A). The cI repressor assay has been successfully used to demonstrate that the T2SS XcpR and T4SS VirB4 and VirB11 proteins oligomerize (11-13).

TOXCAT. The TOXCAT assay is based on the characteristics of the *Vibrio cholerae* ToxR regulator: a strict dimerization-dependent transcriptional activator constituted of a N-terminal helix-turn-helix DNA binding domain, and a C-terminal dimerization domain. The construct consists to a fusion in which the TMH is inserted between the monomeric ToxR DNA-binding domain and the MalE periplasmic protein (Figure 1B). By supporting growth on maltose-minimal media, MalE allows to verify that the TMH is properly inserted. TMH-mediated ToxR dimerization induces binding of ToxR on its operator sequence allowing transcription of a reporter gene. In the initial ToxR system, the reporter gene is *lacZ* (**14**), while the TOXCAT assay uses the *cat* gene (**15**) (Figure 1B). Hence, dimerization of the TMH could be then assessed by measuring the β -galactosidase and chloramphenicol acetyltransferase (resistance to chloramphenicol) activities, respectively (**16**). The TOXCAT assay has been successfully used to provide evidence that the TMH of the T4SS VirB10 subunit oligomerizes (**5**). Further improvements of the ToxR and TOXCAT assays have been published (**17-19**).

1.2. Monitoring TMH heterotypic interactions.

Methods to test heterodimerization of transmembrane helices are based on the two-hybrid reporter fusion approach.

GALLEX. The GALLEX assay is based on the reconstitution of a dimeric LexA transcriptional activator by two interacting TMH. The construct consists to a fusion in which each TMH is inserted between the monomeric LexA N-terminal DNA-binding domain and the MalE periplasmic protein. The elegant improvement is that one of the two TMH is fused to the wild-type LexA N-terminal domain, whereas the second TMH is fused to a LexA N-terminal domain variant bearing a mutation in the DNA-binding motif (LexA408), allowing recognition of a different operator sequence (op408). Formation of helix heterodimers

induces binding of LexA on a dual operator sequence allowing repression of a reporter gene (20-22) (Figure 2A).

BACTH. The bacterial two-hybrid assay (BACTH) is based on the reconstitution of the adenylate cyclase activity conferred by the T18 and T25 domains of the *Bordetella pertussis* Cya protein (23-25) (Figure 2B). Widely used for testing interactions between soluble domains or proteins in multiprotein complexes such as the divisome or secretion systems (26-34), it has only been scarcely used for the study of transmembrane helix-helix interactions (35-37). A detailed protocol for the bacterial two-hybrid assay by Ladant is described in Chapter XX. In this chapter, we provide protocols for the TOXCAT and GALLEX assays.

2. Material

2.1. Monitoring TMH homotypic interactions: the TOXCAT assay.

1. pccan vector (15) (see Note 1).
2. *Escherichia coli* NT326 or MM39 bacterial strains (15).
3. Lysogeny broth (LB) medium: Dissolve 10 g of tryptone, 5 g of yeast extracts and 10 g of NaCl in 1 L of distilled water. Autoclave for 15 min at 121°C. For LB agar plates, add 15 g of bacto-agar prior to autoclave.
4. M9-maltose medium: Dissolve 0.6 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.3 g of $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 50 mg of NaCl, 100 mg of NH_4Cl and 1.5 g of bacto-agar in 90 mL of distilled water. Autoclave. Add 100 mg of casaminoacids, 400 mg of maltose, 25 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 mg of CaCl_2 .
5. Ampicillin stock solution (250×): 25 mg/mL Ampicillin. Dissolve 250 mg of ampicillin in 10 mL of distilled water. Filter sterilize. Store at 4°C.

6. Chloramphenicol stock solution: Dissolve 90 mg of chloramphenicol in 1 mL of absolute ethanol. Store at -20°C.
7. 2.5 mM chloramphenicol solution: Dissolve 8.1 mg of chloramphenicol in 10 mL of ethanol.
8. SDS-PAGE loading buffer: 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue.
9. Lysis buffer: 25 mM Tris-HCl, 2 mM EDTA, pH8.0. Dissolve 303 mg of Tris(hydroxymethyl) aminomethane and 58 mg Ethylene diamine tetraacetic acid (EDTA, disodium salt) in 100 mL of sterile distilled water. Adjust pH to 8.0.
10. Reaction buffer: 100 mM Tris-HCl, pH7.8, 0.1 mM acetyl-CoA, 0.4 mg/mL 5,5'-dithiobis-(2 nitrobenzoic acid) (dTNB). Dissolve 121 mg of Tris, 0.81 mg of acetyl-CoA and 4 mg of dTNB in 10 mL of sterile distilled water. Adjust the pH to 7.8 with HCl.
11. 10-mm filter paper disk.
12. 96-well microplates.
13. Anti-MBP antibodies for MalE immunodetection.
14. Incubator.
15. Spectrophotometer.
16. Labtop centrifuge
17. Water bath at 96°C.
18. Mini-gel caster system and SDS-PAGE apparatus.
19. Protein blotting apparatus
20. Sonifier
21. Microplate reader.

2.2. Monitoring TMH heterotypic interactions: the GALLEX assay.

1. pAML100, pAML108, pBML100 and pBML108 vectors (**20**).
2. *Escherichia coli* NT326 or MM39 bacterial cells (**15**).
3. *Escherichia coli* SU202 bacterial strain (**20**).
4. Lysogeny broth (LB) medium: *see* section 2.1.
5. M9-maltose medium: *see* section 2.1.
6. Ampicillin stock solution (250×): *see* section 2.1.
7. Chloramphenicol stock solution (1000×). 40 mg/mL chloramphenicol. Dissolve 400 mg of chloramphenicol in 10 mL of ethanol. Filter sterilize. Store at 4°C.
8. IPTG stock solution (500×). 0.1 M IPTG. Dissolve 238 mg of Isopropyl-β-D-thiogalactopyranoside (IPTG) in 10 mL of sterile distilled water. Filter sterilize. Store at 4°C.
9. X-Gal stock solution (1000×). 40 mg/mL X-Gal. Dissolve 40 mg of 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) in 1 mL of dimethylformamide. Prepare extemporarily and do not store.
10. 0.1 % SDS: Dissolve 50 mg of sodium dodecylsulphate (SDS) in 50 mL of distilled water.
11. Chloroform.
12. ONPG stock solution. 4 mg/mL ortho-nitrophenyl-β-D-galactopyranoside (ONPG). Dissolve 20 mg of ONPG in 5 mL of buffer Z.
13. SDS-PAGE loading buffer: 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue.
14. Buffer Z. Dissolve 2.15 g of Na₂HPO₄•12 H₂O, 0.29 g of Na₂HPO₄•H₂O, 75 mg of KCl and 25 mg of MgSO₄•7H₂O in 100 mL of distilled water. Adjust the pH to 7.0. Add 270 μL of β-mercapthoethanol. Prepare extemporarily and do not store.
15. Anti-MBP antibodies for MalE immunodetection.

16. 96-well microplates.
17. Incubator.
18. Spectrophotometer.
19. Labtop centrifuge
20. Water bath at 96°C.
21. Mini-gel caster system and SDS-PAGE apparatus.
22. Protein blotting apparatus
23. Microplate reader.

3. Methods

3.1. Monitoring TMH homotypic interactions: the TOXCAT assay.

1. Clone the DNA fragment corresponding to the TMH to be studied into the pccan vector to yield a plasmid producing the ToxR'-TMH-MalE fusion protein. Before testing the homodimerization of the TMH, verify that your fusion protein is properly produced (steps 3-8) and inserted in the inner membrane (steps 9-10). The dimerization of the TMH is then assessed by the disk diffusion assay (steps 11-16) and quantified by measuring the chloramphenicol acetyltransferase activity (steps 17-26).
2. Transform the empty pccan vector and your pccan construct into NT326 or MM39 *E. coli* competent cells. Select on LB-ampicillin plates (*see Note 1*).
3. Pick a single colony of each transformation and grow cells in 20 mL of LB medium supplemented with ampicillin (100 µg/mL) until an optical density at 600 nm (OD₆₀₀) of 0.8.
4. Harvest 2 mL of cells by centrifugation at 4,000 × *g* for 5 min.
5. Discard the supernatants and resuspend the cell pellets into 20 µL of SDS-PAGE loading buffer.

6. Boil the samples for 10 min at 96°C.
7. Separate proteins by SDS-PAGE and transfer onto nitrocellulose membrane using your favorite protocol.
8. Use western-blotting to immuno-detect your fusion protein using commercial anti-MalE (anti-MBP) antibodies.
9. Streak 20 µL of the bacterial culture obtained after step 3 of section 3.1 onto M9-Maltose medium.
10. After incubation for 48 h at 37°C, verify that your strain grew on M9-maltose medium.
11. Drop a 10-mm filter paper disk at the centre of a LB-ampicillin plate (*see Note 2*).
12. Add 60 µL of the chloramphenicol stock solution (90 mg/mL) on the filter paper disk.
13. Incubate LB plates with chloramphenicol-disks for 6 h at 37°C.
14. Remove the disk.
15. Spread 2 mL of the culture obtained at step 3 of section 3.1 on the LB-ampicillin plate to make a lawn. Eliminate the excess of culture.
16. After incubation for 16 h at 37°C, measure the halo of chloramphenicol sensitivity (*see Note 3*).
17. Centrifuge 3 mL of the culture obtained at step 3 of section 3.1 at 4,000 × g for 5 min. (in triplicate).
18. Discard the supernatant and resuspend the cell pellet in 500 µL of Lysis buffer. Vortex.
19. Lyse the cells by sonication using a sonifier.
20. Clear the lysate by centrifugation at 10,000 × g for 15 min.
21. In a 96-well microplate, mix 15 µL of the cleared lysate with 220 µL of Reaction buffer.
22. Measure the absorbance at 412 nm (A_{412} ; *see Note 4*) and at 550 nm (A_{550} ; cell debris) every 20 sec for 4 min using a microplate reader.
23. Inject 15 µL of 2.5 mM chloramphenicol in each well.

24. Measure the absorbance at 412 nm (*see Note 4*) and at 550 nm (cell debris) every 20 sec for 10 min using a microplate reader.
25. Divide each A_{412} value by the corresponding A_{550} value and plot these values against time.
26. Calculate the chloramphenicol acetyltransferase activity based on the slope in the linear part of the curve (initial rate).

3.2. Monitoring TMH heterotypic interactions: the GALLEX assay.

1. Clone the DNA fragment corresponding to the first TMH to be studied (TMH1) into the pAML100 vector to yield a pACYC184 derivative plasmid producing the LexA_{WT}'-TMH1-MalE fusion protein. Clone the DNA fragment corresponding to the second TMH to be studied (TMH2) into the pBML108 vector to yield a pBR322 derivative plasmid producing the LexA₄₀₈'-TMH2-MalE fusion protein. Before testing the heterodimerization of the TMH, verify that your fusion protein is properly produced (steps 3-8) and inserted in the inner membrane (steps 9-10). The dimerization of the TMH is then assessed on LB-X-Gal plates (steps 11-14) and quantitated by measuring the β -galactosidase activity (steps 15-22).
2. Transform the empty pAML100 and pBML108 vectors as well as the pAML100-TMH1 and pBML108-TMH2 constructs into NT326 or MM39 *E. coli* competent cells. Select on LB plates supplemented with ampicillin (pBML108 derivatives) or chloramphenicol (pAML100 derivatives).
3. Pick a single colony of each transformation and grow cells in 3 mL of LB medium supplemented with IPTG and ampicillin or chloramphenicol until OD₆₀₀ of 0.8.
4. Harvest 2 mL of cells by centrifugation at 4,000 $\times g$ for 5 min.
5. Discard the supernatants and resuspend the cell pellets into 20 μ L of SDS-PAGE loading buffer.

6. Boil the samples for 10 min at 96°C.
7. Separate proteins by SDS-PAGE and transfer onto nitrocellulose membrane using your favorite protocol.
8. Use western-blotting to immuno-detect your fusion protein using commercial anti-MalE (anti-MBP) antibodies.
9. Streak 20 μ L of the bacterial culture obtained after step 3 of section 3.2 onto M9-maltose medium.
10. After incubation for 48 h at 37°C, verify that your strain grew on M9-maltose medium.
11. Co-transform the pAML100 and pAML100-TMH1 vectors in combination with the pBLM108 and pBLM108-TMH2 vectors into SU202 *E. coli* competent cells (*see Note 5*). Select on LB plates supplemented with ampicillin and chloramphenicol.
12. Pick a single colony of each transformation and grow cells in 3 mL of LB medium supplemented with IPTG, ampicillin and chloramphenicol until an OD₆₀₀ of 0.8.
13. Drop 15 μ L of the bacterial culture obtained after step 12 of section 3.2 onto LB plates supplemented with IPTG, ampicillin, chloramphenicol and X-Gal.
14. After 6, 14 and 24 h of incubation at 37°C, observe the coloration of the spots. White spots correspond to strains with no β -galactosidase activity (no interaction), whereas blue spots correspond to strains with β -galactosidase activity (interaction) (*see Note 6*).
15. Mix 200 μ L of the bacterial culture obtained after step 12 of section 3.2 with 800 μ L of buffer Z into a 1.5-mL eppendorf tube. Vortex.
16. Add one drop of 0.1% SDS and 2 drops of chloroform to lyse cells. Vortex for 10 sec.
17. In a 96-well microplate, mix 50 μ L of the cleared lysate with 150 μ L of buffer Z.
18. Measure the absorbance at 420 nm (wavelength of orthonitro-phenol) and at 550 nm (A_{550} ; cell debris) every 30 sec for 2 min using a microplate reader.
19. Inject 40 μ L of the ONPG solution in each well.

20. Measure the absorbance at 420 nm and at 550 nm every 30 sec for 20 min using a microplate reader.
21. Divide each A_{420} value by the corresponding A_{550} value and plot these values against time.
22. Calculate the β -galactosidase activity based on the slope in the linear part of the curve (initial rate).

4. Notes

1. pccan is a vector comprising the sequence corresponding to the ToxR N-terminal domain and that corresponding to Male separated by a multiple cloning site allowing insertion of the sequence corresponding to the TMH of interest. Positive and negative controls have been developed by Russ and Engelman corresponding to the wild-type and mutated TMH of the glycophorin A, respectively (15).
2. Use three LB-ampicillin plates per strain to be tested.
3. The diameter of the halo reflects the ability of the strain to resist chloramphenicol, and therefore is directly and inversely linked to the expression of the *cat* gene that is induced by the TMH dimerization. If the TMH dimerizes, the expression level of *cat* is high and hence the diameter of the halo is small.
4. The reaction catalyzed by the chloramphenicol acetyltransferase consists to the acetylation of the chloramphenicol, and the release of free co-enzyme A. Co-enzyme A then reacts with the 5,5'-dithiobis-(2-nitrobenzoic acid), resulting in an increase of the absorbance at 412 nm.
5. You should obtain the combinations pAML100+pBLM108, pAML100+pBLM108-TMH2, pAML100-TMH1+pBLM108 and pAML100-TMH1+pBLM108-TMH2.

6. MacConkey/maltose could be used as reporter medium instead of LB-X-Gal plates. In case of use of MacConkey/maltose plates, the coloration of the spots differs: yellow spots correspond to strains with no β -galactosidase activity (no interaction), whereas red spots correspond to strains with β -galactosidase activity (interaction).

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Figure Legends

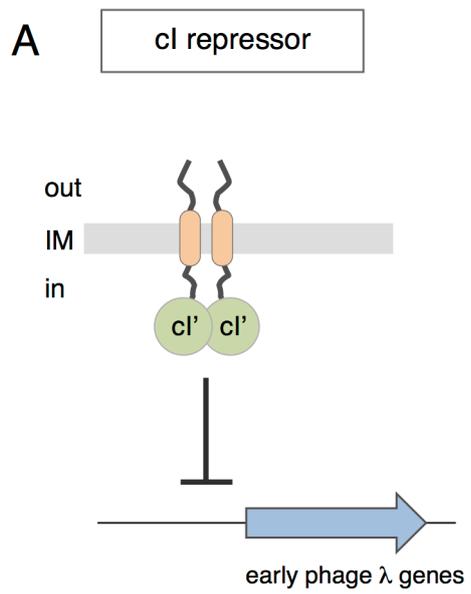
Figure 1. Schematic representation of the assays for monitoring TMH homotypic

interactions. (A) cI repressor assay. The TMH of interest (orange) is fused to the cI DNA-binding domain (green). Reconstitution of the cI dimer results in the repression of early phage λ genes (blue). Repression of phage λ genes confers protection against phage λ

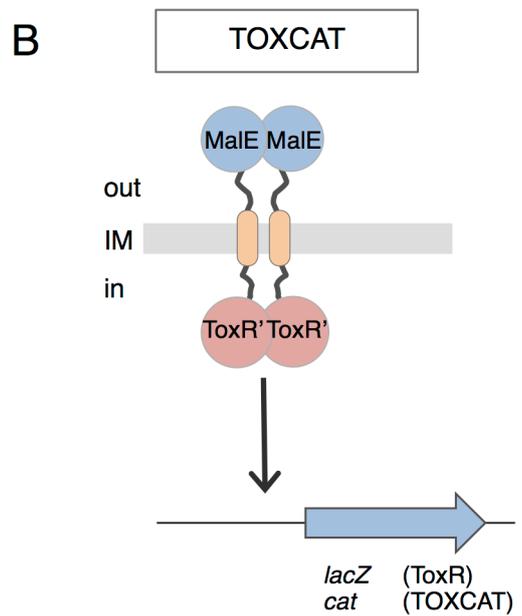
infection. (B) ToxR and TOXCAT assays. The TMH of interest (orange) is fused between the *Vibrio cholerae* ToxR DNA-binding domain (red) and the MalE protein (blue).

Reconstitution of the ToxR dimer results in the expression of the reporter genes (blue, *lacZ* for the ToxR assay, *cat* for the TOXCAT assay).

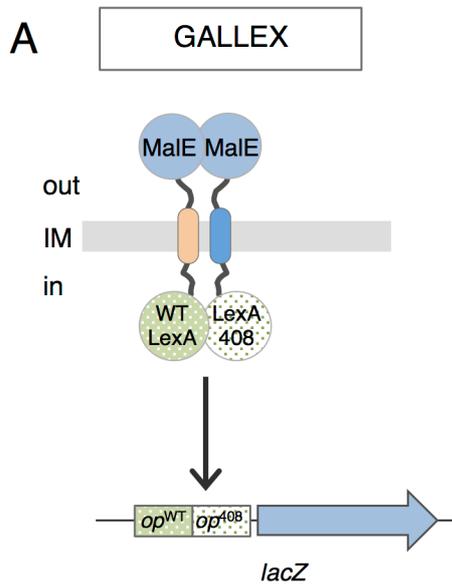
Figure 2. Schematic representation of the assays for monitoring TMH heterotypic interactions. (A) GALLEX assay. The first TMH of interest (orange) is fused between the wild-type LexA DNA-binding domain (WT LexA) and MalE whereas the second TMH (blue) is fused between the LexA⁴⁰⁸ variant (LexA 408) and MalE. Reconstitution of the LexA^{WT}/LexA⁴⁰⁸ dimer results in the expression of the reporter gene (blue). (B) BACTH assays. The first TMH of interest (orange) is fused to the T18 domain of the *B. pertussis* adenylate cyclase whereas the second TMH (blue) is fused to the T25 of adenylate cyclase. Reconstitution of the T18/T25 adenylate cyclase results in the production of cAMP. Binding of cAMP to the catabolite activator protein (CAP) induces the expression of the reporter gene (blue).



no interaction: sensitive to phage λ infection
 interaction: resistant to phage λ infection

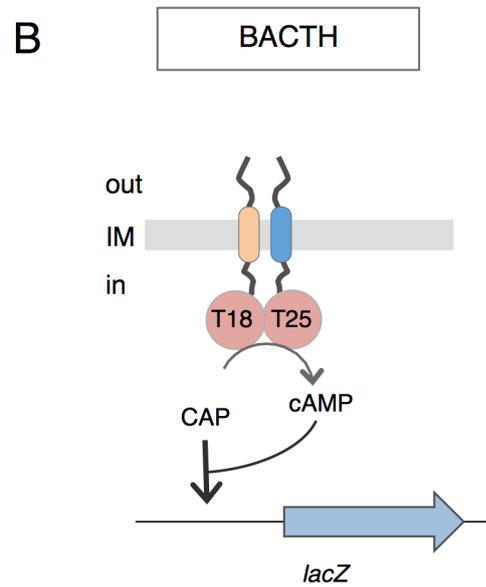


no interaction: no β -galactosidase activity (ToxR)
 sensitive to chloramphenicol (TOXCAT)
 interaction: β -galactosidase activity (ToxR)
 resistant to chloramphenicol (TOXCAT)



no interaction: no β -galactosidase activity

interaction: β -galactosidase activity



no interaction: no β -galactosidase activity

interaction: β -galactosidase activity