ORIGINAL ARTICLE

A novel assay of bacterial peptidoglycan synthesis for natural product screening

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Although a large number of microbial metabolites have been discovered as inhibitors of bacterial peptidoglycan biosynthesis, only a few inhibitors were reported for the pathway of UDP-MurNAc-pentapeptide formation, partly because of the lack of assays appropriate for natural product screening. Among the pathway enzymes, D-Ala racemase (Alr), D-Ala:D-Ala ligase (Ddl) and UDP-MurNAc-tripeptide:D-Ala-D-Ala transferase (MurF) are particularly attractive as antibacterial targets, because these enzymes are essential for growth and utilize low-molecular-weight substrates. Using dansylated UDP-MurNAc-tripeptide and L-Ala as the substrates, we established a cell-free assay to measure the sequential reactions of Alr, Ddl and MurF coupled with translocase I. This assay is sensitive and robust enough to screen mixtures of microbial metabolites, and enables us to distinguish the inhibitors for D-Ala–D-Ala formation, MurF and translocase I. D-cycloserine, the D-Ala-D-Ala pathway inhibitor, was successfully detected by this assay (IC$_{50}$=1.7 µg ml^{-1}). In a large-scale screening of natural products, we have identified inhibitors for D-Ala–D-Ala synthesis pathway, MurF and translocase I.
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INTRODUCTION

The emergence of drug-resistant bacteria is an unavoidable problem in anti-infectious therapy. One of the strategies to overcome this problem is to find new anti-bacterial agents towards novel targets. Bacterial peptidoglycan synthesis is a rich source of such targets, because most enzymes in this pathway are unique in bacteria and essential for growth.[1–4] In fact many antibiotics, including the clinically important beta-lactam and glycopeptides antibiotics, are peptidoglycan synthesis inhibitors. Although most of these antibiotics target the later stage of peptidoglycan synthesis, such as transpeptidation and/or transglycosylation steps, only a few inhibitors are known for enzymes involved in the earlier stage of UDP-MurNAc-pentapeptide formation. In the cytoplasm, the D-Ala–D-Ala dipeptide is synthesized from L-Ala by pyridoxal phosphate-dependent D-alanine racemase (Alr) and ATP-dependent D-Ala:D-Ala ligase (Ddl). The dipeptide is transferred to UDP-MurNAc-tripeptide (UDP-MurNAc-L-Ala-γ-D-Glu-m-DAP) by ATP-dependent D-Ala–D-Ala adding enzyme UDP-MurNAc-tripeptide:D-Ala–D-Ala transferase (MurF) to form UDP-MurNAc-pentapeptide. Then, phospho-N-acetylmuramyl-pentapeptide translocase (translocase I, MraY) catalyzes the transfer of the MurNAc-pentapeptide moiety to the lipid carrier undecaprenyl phosphate to form lipid I. To date, a variety of nucleoside antibiotics have been reported as translocase I inhibitors,[5] such as mureidomycins,[6] pacidamycins,[7] napsamycins,[8] liposidomycins,[9] tunicamycin,[10] capuramycins,[11–17] muraymycins[18] and caprazamycins.[19,20] In contrast, only a few inhibitors have been reported for the steps of UDP-MurNAc-pentapeptide formation. D-cycloserine and O-carbamyl-D-serine are known inhibitors for D-Ala–D-Ala pathways Alr and Ddl,[21–23] and recently a few synthetic compounds were reported as MurF inhibitors.[24] To date, there are some reports on the assay method for D-Ala–D-Ala pathway[25,26] and MurF,[27,28] but none of them are high-throughput functional assays appropriate for large-scale natural product screenings. In this paper, we describe the development of a cell-free assay to measure the sequential reactions of D-Ala–D-Ala formation and MurF coupled with translocase I. To prepare sufficient amount of the substrate dansylated UDP-MurNAc-tripeptide, we also established a large-scale fermentation protocol of UDP-MurNAc-tripeptide. With this method, we carried out a high-thoughput screening of natural products, whose result is also presented.

MATERIALS AND METHODS
Chemicals
Undecaprenyl phosphate was purchased from Larodan Fine Chemicals (Malmo, Sweden). D-cycloserine was purchased from Sigma (St Louis, MO, USA).

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Plasmid construction

The alr, ddlA, murF and mraY genes were amplified by PCR using *Escherichia coli* K-12 DNA as a template. These products were digested with *Bam*HI, *Hin*dIII and cloned between the corresponding sites of the pET28a plasmid vector (Novagen, Madison, WI, USA). In this construct, each gene is expressed under the control of an IPTG-inducible promoter with an N-terminal His$_6$ extension.

Enzyme preparation

E. coli BL21(DE3) harboring a recombinant plasmid with alr, ddl or murF was grown at 37 °C in 2×YT medium containing 25 μg ml^{-1} of kanamycin. At an A_{600} of 0.6, IPTG was added at a final concentration of 1 mM, and incubation was continued for 6 h at 37 °C with shaking. The cells harvested by centrifugation were washed with 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM MgCl$_2$, and re-suspended in the same buffer at 4 °C. After disruption of the cells by sonication, the remaining cells were removed by centrifugation (5000 g, 10 min) and the supernatant was adsorbed onto the Ni-NTA-agarose column (QIAGEN, Germantown, MD, USA). Elution was carried out at 4 °C first with 50 mM Tris-HCl buffer (pH 8.0), and thereafter eluted with 50 mM Tris-HCl buffer (pH 8.0) and 200 mM imidazole. The purified enzyme solutions were stored at −80 °C.

Translocase I was prepared as follows: *E. coli* strain C43 (DE43) harboring recombinant plasmid pET-mraY was grown at 37 °C in 2×YT medium containing 25 μg ml^{-1} of kanamycin. At an A_{600} of 0.7, IPTG was added at a final concentration of 1 mM, and incubation was continued for 16 h at 25 °C with shaking. The cells harvested by centrifugation were washed with 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM MgCl$_2$, and re-suspended in the same buffer at 4 °C. After disruption of the cells by sonication, the remaining cells were removed by centrifugation (5000 g, 10 min), and membrane fragments were collected by ultracentrifugation (105 000 g, 60 min) at 4 °C. The pellet was washed with the same buffer and re-suspended in solubilization buffer consisting of 50 mM Tris-HCl buffer (pH 8.0), 0.1 mM MgCl$_2$, 1% Triton X-100 and 30% (v/v) glycerol. The suspension was stirred for 30 min at 4 °C to solubilize the enzyme. After removal of insoluble materials by ultracentrifugation using the same conditions as described above, the resulting crude enzyme solution was stored at −80 °C.

Preparation of UDP-MurNAc-tripeptide

Based on the method reported earlier,[29] we established a large-scale fermentation of UDP-MurNAc-tripeptide. *Bacillus cereus* SANK70880 was inoculated into each of twelve 2-l Erlenmeyer flasks containing 500 ml of the medium consisting of bactopeptone 0.5%, yeast extract 0.5%, meat extract 0.5%, glucose 0.2%, KH$_2$PO$_4$ 0.2% and CB-442 0.01% (NOF Co., Ltd, Tokyo, Japan), and was incubated on a rotary shaker (210 rpm) at 37 °C for 24 h. Then 6 l of the culture was transferred into a 600-l tank containing 300 l of the same medium as mentioned above. The fermentation condition is as follows: temperature 37 °C, initial aeration rate 1 vvm, stirrer speed 83 rpm, dissolved oxygen 5 p.p.m., and pressure 100 kPa. After 2 h of fermentation (A_{600}=0.85), D-cycloserine (final concentration 100 μg ml^{-1}) was added. Cells were harvested by centrifugation for 30 min after addition of D-cycloserine. The pellet (515 g) was washed, suspended in 1-l phosphate buffer, and boiled at 100 °C for 15 min. After centrifugation at 9000 rpm for 30 min, the supernatant was adsorbed onto Sephadex A-25 (2.5 l). The column was washed with 100 mM KCl (12 l) and eluted with 300 mM KCl (20 l). The eluate was adjusted to pH 2.0 with 1 M HCl and adsorbed onto a SEPABEADS SP207 column (2.5 l). The column was washed with 0.01 M HCl (10 l) and water (2 l), and eluted with 20% MeOH (10 l). The eluate was lyophilized to give a powder of UDP-MurNAc-tripeptide (3.8 g) with >95% purity as confirmed by HPLC.

Dansylation of UDP-MurNAc-tripeptide

UDP-MurNAc-tripeptide (5 μmol) was dissolved in a 1:1 (v/v) mixture (150 ml) of 0.25 M NaHCO$_3$ and ME$_2$CO, and dansyl chloride (210 μmol) was added. The solution was stirred for 4 h at room temperature in the dark. The Me$_2$CO was evaporated off and the aqueous solution was chromatographed on a Bio-Gel P2 column (Bio-Rad, Hercules, CA, USA) with water. Fractions containing UDP-MurNAc-dansyltripeptide were identified using HPLC and lyophilized. UDP-MurNAc-dansyltripeptide was stored at −20 °C.

Translocase I-coupled fluorescence-based assay

MurF and translocase I coupling reaction was carried out in a 96-well microtiter polystyrene plate (Corning Coaster, Corning, NY, USA, #3694). The assay mixture contains 100 mM Tris-HCl (pH 8.6), 50 mM KCl, 25 mM MgCl$_2$, 0.2% Triton X-100, 37.5 μM undecaprenyl phosphate, 100 μM UDP-MurNAc-dansyltripeptide, 250 μM D-Ala–D-Ala, 250 μM ATP and 8% glycerol. The reaction was initiated by the addition of 10 μl (5 μg) of MurF and translocase I (1–4 μg) mixture, and monitored by measuring the increase of fluorescence at 538 nm (excitation at 355 nm). The Alr, Ddl, MurF and translocase I coupling reaction was carried out as described above, except that 150 μM L-Ala was used instead of D-Ala–D-Ala, and a four-enzyme mixture (10 μl) including Alr (0.5 μg) and Ddl (0.5 μg). The assay condition in 384-well plate format was the same as that in the 96-well plate format described above, except that the assay volume was half of that in the 96-well plate format and reaction time in the screening mode was 60 min.

HPLC assay

HPLC detection of the MurF activity was carried out as described by Duncan *et al.*[30] with slight modifications. The reaction mixture containing 100 mM Tris-HCl (pH 8.6), 40 mM KCl, 10 mM MgCl$_2$, 200 μM UDP-MurNAc-tripeptide, 200 μM D-Ala–D-Ala, 200 μM ATP and 8% glycerol was added to 10 μl of MurF and incubated. The reaction was stopped by boiling at 100 °C for 3 min, and after being centrifuged at 11 000 rpm for 10 min, the supernatant was analyzed by HPLC with Symmetry C18 column (eluent 3% acetonitrile–0.3% TEAP aq. (pH 3.0); flow rate 1.0 ml min^{-1}; detection 260 nm).

RESULTS AND DISCUSSION

Fluorescence-based determination of MurF activity

Brandish *et al.*[31] reported the assay to measure translocase I activity by determining the difference in fluorescent intensities of the substrate UDP-MurNAc-dansylpentapeptide and the product dansylated lipid I. We applied this method to high-throughput screening of natural products and discovered novel translocase I inhibitors.[13–17,32–35] As the assay is sensitive and robust enough, we tried to measure MurF activity by coupling with this reaction (Figure 1). For that purpose, we first established a large-scale fermentation protocol for UDP-MurNAc-tripeptide to prepare the labeled substrate UDP-MurNAc-dansyltripeptide. From a 300-l culture of D-cycloserine-treated *Bacillus cereus*, we obtained 3.8 g of UDP-MurNAc-tripeptide with >95% purity. Then we used UDP-MurNAc-tripeptide or UDP-MurNAc-dansyltripeptide as the substrate for the detection of MurF activity. In cell-free MurF assay, each substrate was converted to the corresponding product, UDP-MurNAc-pentapeptide or UDP-MurNAc-dansylpentapeptide, which was confirmed by HPLC analysis.[30] This indicated that UDP-MurNAc-dansyltripeptide was used as the substrate of MurF, and suggested that MurF activity could be measured in a high-throughput mode by coupling translocase I.

To exemplify this, we used D-Ala–D-Ala and UDP-MurNAc-dansyltripeptide as the substrates instead of UDP-MurNAc-dansylpentapeptide for the translocase I reaction. We observed a small increase of the fluorescence intensity without MurF, but in the presence of MurF the fluorescence intensity was dramatically increased (Figure 2), which suggested that MurF and translocase I reactions were efficiently coupled in this assay condition. The reaction was also dependent on D-Ala–d-Ala as expected. The minor increase of the fluorescence without MurF was reduced in the absence of translocase I (Figure 2), suggesting that dansylated tripeptide could be incorporated into lipid I by translocase I.

Detemination of Alr and Ddl activity

Next, we incorporated the D-Ala–D-Ala biosynthesis pathway in this coupling reaction by adding L-Ala instead of D-Ala–D-Ala in
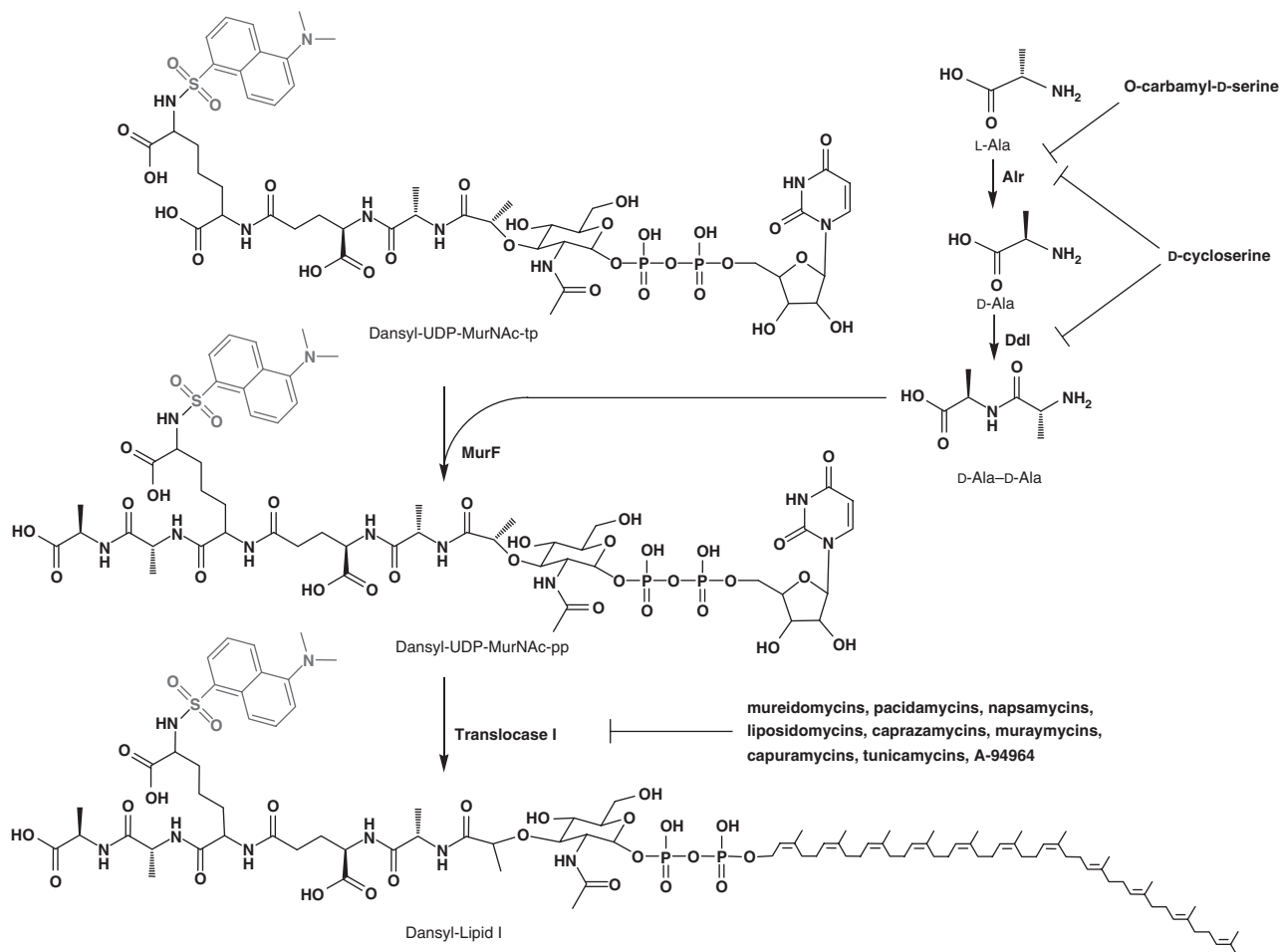
Figure 1 Fluorescence-based assay of D-Ala–D-Ala pathway, MurF and translocase I. In bacterial peptidoglycan biosynthesis, the UDP-MurNAc-pentapeptide is synthesized from L-Ala and UDP-MurNAc-tripeptide by Alr, Ddl and MurF. Then, translocase I converts UDP-MurNAc-pentapeptide to lipid I. A novel assay method to measure the sequential reactions of Alr, Ddl and MurF coupled with translocase I was designed by using dansylated UDP-MurNAc-tripeptide as the substrate. In this assay, the fluorescence intensity was expected to increase by enzymatic reactions.
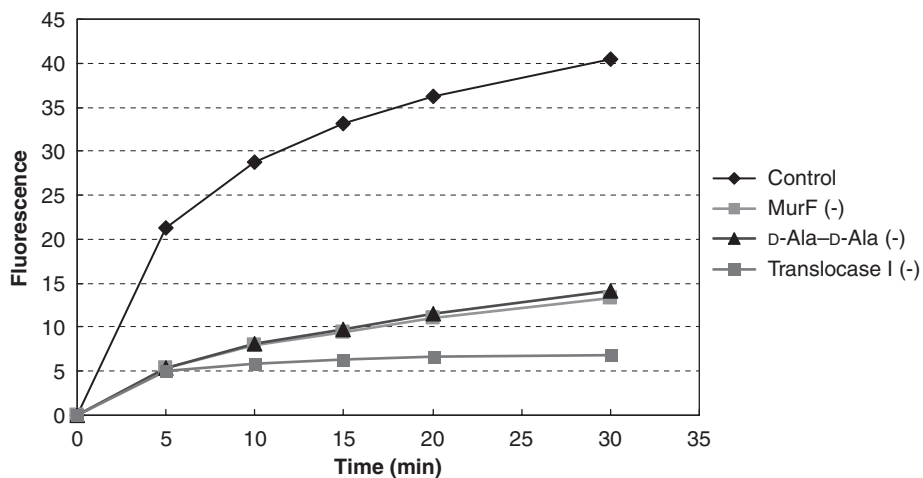

Figure 2 Time course of MurF and translocase I coupling reaction. MurF and translocase I coupling reaction was initiated by the addition of MurF and translocase I enzyme into the assay mixture described in 'Materials and methods', and monitored by measuring the increase of fluorescence at 538 nm (excitation at 355 nm).
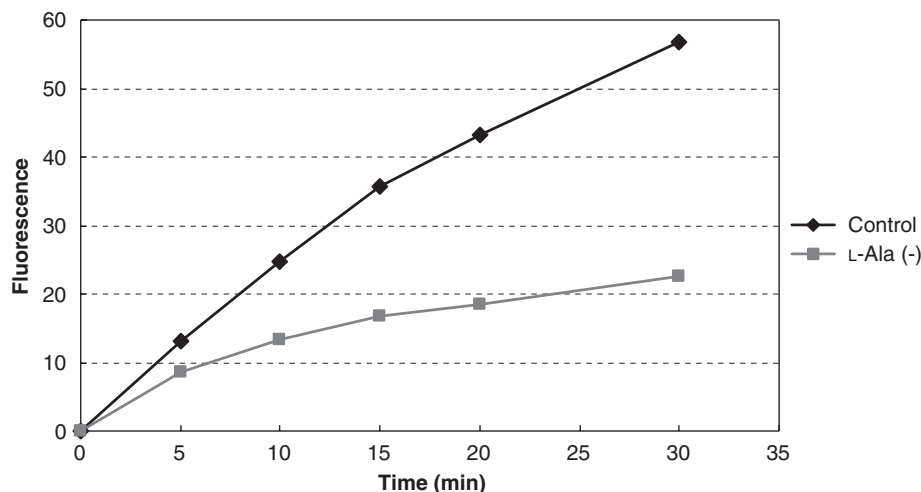
Figure 3 Time course of Alr, Ddl, MurF and translocase I coupling reaction. Alr, Ddl, MurF and translocase I coupling reaction was carried out as shown in Figure 2, except that 150 μm L-Ala was used instead of D-Ala–D-Ala, and a four-enzyme mixture instead of the two.
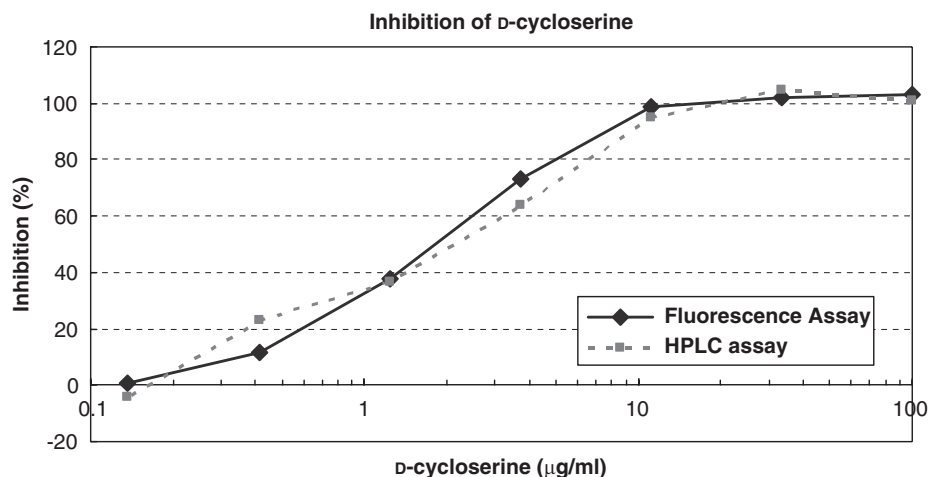

Figure 4 Assay validation by D-cycloserine. The inhibitory activity of D-cycloserine for Alr, Ddl, MurF and translocase I coupling reaction was checked by both HPLC and fluorescence-based assay.

the assay mixture and also by supplying Alr and Ddl enzymes. The fluorescence intensity was increased largely depending on L-Ala (Figure 3). We confirmed the contribution of D-Ala–D-Ala pathway in this reaction by checking the inhibitory activity of D-cycloserine (Figure 4). The IC_{50} value of D-cycloserine was estimated as $1.7\,\mu g\,ml^{-1}$ ($=17\,\mu M$), which is compatible with that obtained in the HPLC assay.[25]

Screening of microbial metabolites for inhibitors of UDP-MurNAc-pentapeptide formation

Our goal is to obtain inhibitors for Alr, Ddl and MurF from microbial metabolites using this assay. We optimized the assay condition in 384-well plate format with Z' values in the range from 0.6 to 0.8 (Figure 5), and carried out a large-scale screening of microbial metabolites (actinomycetes, fungi and bacteria metabolites). On screening some samples showed positive activities (hit rate approximately 0.5%). After retesting these samples with initial fluorescence-based screening, we

checked their inhibitory activities by HPLC assay with all four enzymes. Next, these hit samples were tested in the translocase I reaction as well as in the MurF plus translocase I reaction, and categorized according to the inhibitory activities in these three assay conditions. We identified the translocase I inhibitors reported earlier, such as capuramycins, mureidomycins, liposidomycins and tunicamycins (data not shown). In addition, we found F-11334A1 as an inhibitor of the D-Ala–D-Ala pathway ($IC_{50}=20\,\mu M$), and (−)-epigallocatechin gallate and pleurotin as MurF inhibitors ($IC_{50}=9\,\mu M$ and $41\,\mu M$, respectively) (Figure 6). F-11334A1 was earlier reported as an inhibitor of neutral sphingo-myelinase.[36] Pleurotin was discovered as an anti-bacterial substance by Robbin *et al.*[37] and (−)-epigallocatechin gallate was reported to have an anti-bacterial activity.[38] The relationship between the reported activities for these compounds and our current observation remains to be further studied. Nevertheless, the method described here is robust enough to screen a large number of mixture samples in
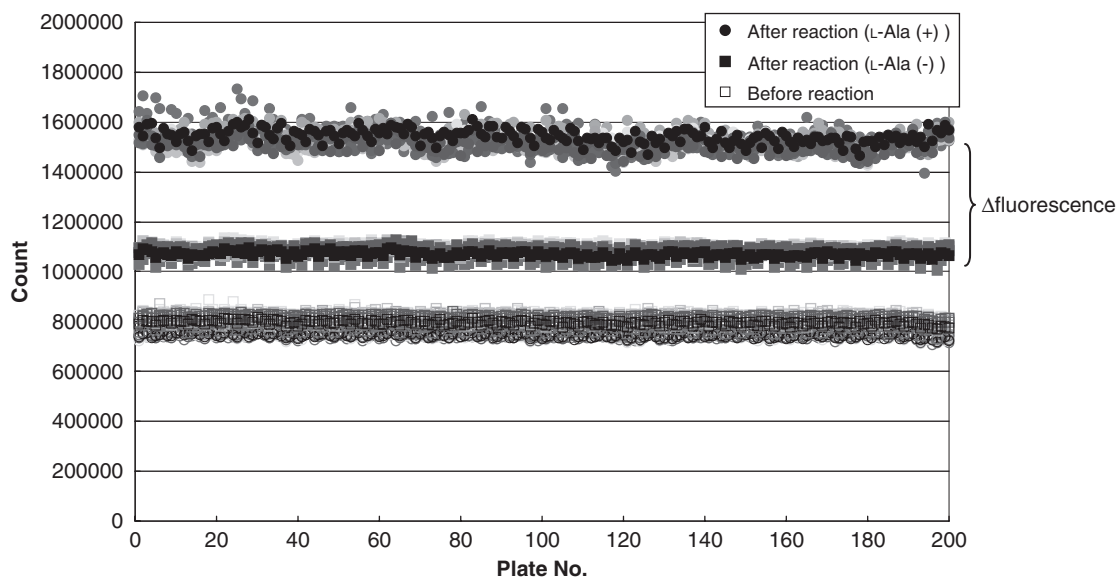
Figure 5 High-throughput screening of D-Ala–D-Ala pathway, MurF and translocase I. Variation among wells in the 384-well plate format was checked. Fluorescence enhancement depending on Alr, Ddl, MurF and translocase I reaction was determined by deducting the count after reaction (L-Ala (−)) from the count after reaction (L-Ala (+)). Therefore, Z' value was derived from data points such that the 'maximum' signal was obtained by deducting the count before reaction from that after reaction (L-Ala (+)) and the 'minimum' signal was obtained by deducting the count before reaction from that after reaction (L-Ala (−)).
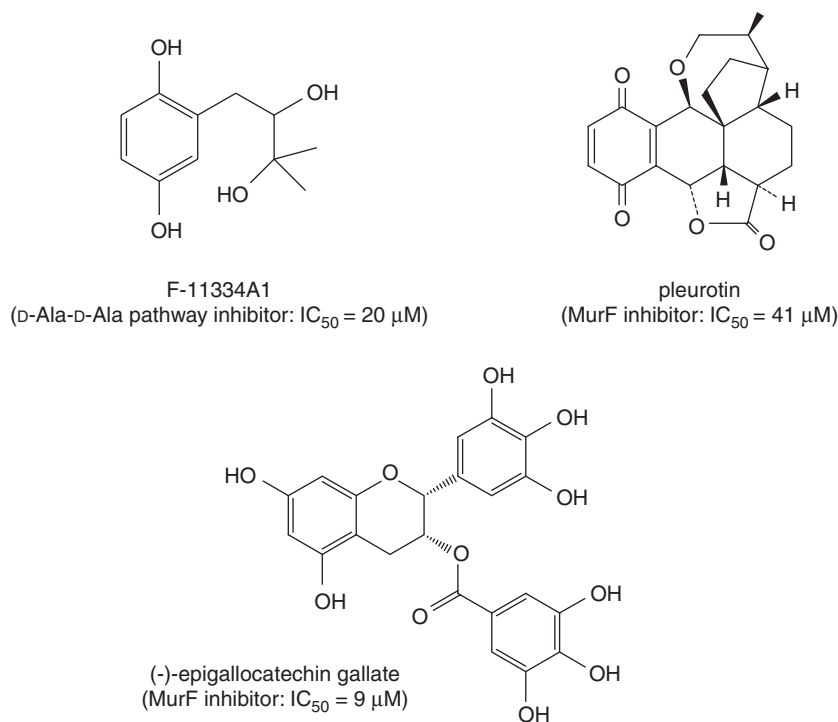

F-11334A1
(D-Ala-D-Ala pathway inhibitor: $IC_{50} = 20\ \mu M$)

pleurotin
(MurF inhibitor: $IC_{50} = 41\ \mu M$)

(-)-epigallocatechin gallate
(MurF inhibitor: $IC_{50} = 9\ \mu M$)

Figure 6 Identification of inhibitors from our screening. Chemical structures and inhibitory activities of inhibitors from our novel fluorescence-based assay of D-Ala–D-Ala pathway, MurF and translocase I are shown.

a high-throughput mode, and will greatly facilitate a discovery of novel antibiotics.

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