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High-Throughput Screen for Inhibitors of Transglycosylase and/or Transpeptidase Activities of *Escherichia coli* Penicillin Binding Protein 1b

B. Chandrakala, Radha K. Shandil, Upasana Mehra,† Sudha Ravishankar, Parvinder Kaur, Veeraraghavan Usha,‡ Bina Joe,§ and Sunita M. deSousa*

AstraZeneca India Pvt. Ltd., Bangalore 560 024, India

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Penicillin binding protein (PBP) 1b of *Escherichia coli* has both transglycosylase and transpeptidase activities, which are attractive targets for the discovery of new antibacterial agents. A high-throughput assay that detects inhibitors of the PBPs was described previously, but it cannot distinguish them from inhibitors of the MraY, MurG, and lipid pyrophosphorylase. We report on a method that distinguishes inhibitors of both activities of the PBPs from those of the other three enzymes. Radioactive peptidoglycan was synthesized by using *E. coli* membranes. Following termination of the reaction the products were analyzed in three ways. Wheat germ agglutinin (WGA)-coated scintillation proximity assay (SPA) beads were added to one set, and the same beads together with a detergent were added to a second set. Type A polyethylenimine-coated WGA-coated SPA beads were added to a third set. By comparison of the results of assays run in parallel under the first two conditions, inhibitors of the transpeptidase and transglycosylase could be distinguished from inhibitors of the other enzymes, as the inhibitors of the other enzymes showed similar inhibitory concentrations (IC_{50}s) under both conditions but the inhibitors of the PBPs showed insignificant inhibition in the absence of detergent. Furthermore, comparison of the results of assays run under conditions two and three enabled the distinction of transpeptidase inhibitors. Penicillin and other β-lactams showed insignificant inhibition with type A beads compared with that shown with WGA-coated SPA beads plus detergent. However, inhibitors of the other four enzymes (tunicamycin, nisin, bacitracin, and moenomycin) showed similar IC_{50}s under both conditions. We show that the main PBP being measured under these conditions is PBP 1b. This screen can be used to find novel transglycosylase or transpeptidase inhibitors.

In the quest for new antibacterial agents, cell wall targets, in particular, the synthesis of peptidoglycan, play an important role. Peptidoglycan is unique to the bacterial cell, has no mammalian counterpart, and is present in most bacterial cell walls, so agents that inhibit its synthesis have the potential to become broad-spectrum antibiotics. In particular, the penicillin binding proteins (PBPs) are attractive targets because of their periplasmic location, which precludes resistance due to drug efflux and problems due to permeability of the membrane.

Peptidoglycan is a polymer of a repeating disaccharide-peptide unit, *N*-acetylglucosamine and *N*-acetyl muramylpentapeptide (MurNAcpp), with the peptide chains attached to adjacent glycan strands being cross-linked. Five enzymes are involved in the late stages of its assembly at the membrane. The MraY and MurG enzymes catalyze synthesis of the disaccharide precursor on the lipid carrier molecule. Subsequently, the disaccharide unit is polymerized by the transglycosylase at the extracellular surface of the membrane and the peptide chains attached to the muramic acid are cross-linked by the transpeptidase (25). The fifth enzyme, the lipid pyrophosphorylase, is involved in recycling of the lipid carrier undecaprenol (29).

Some high-molecular-mass penicillin binding proteins (PBPs), e.g., PBP 1a and PBP 1b of *Escherichia coli*, have dual activities: both transglycosylase and transpeptidase activities (15, 18, 19, 26). These enzymes are the targets of the glycopeptides and the β-lactam antibiotics (23), which have been very successful in the clinic. While the increasing rates of microbial resistance to β-lactams and glycopeptides are a major deterrent to the development of new agents of these classes, a transpeptidase or transglycosylase inhibitor of a novel chemical class would be an attractive candidate for drug development.

However, most assays that measure the transglycosylase or transpeptidase are not easy to perform with high throughput (3, 7, 24, 27). Those that are amenable to high-throughput screening are either not true enzyme assays (30) or cannot distinguish between inhibitors of the transglycosylase or transpeptidase and those of other enzymes that are assayed along with them (4, 5, 8, 17, 24).

In particular, an assay for the transpeptidase is difficult to design because it necessitates differentiation between the substrate, nascent peptidoglycan, and the product, cross-linked peptidoglycan. The two have very similar chromatographic properties and are not easy to distinguish (3, 11, 16, 17). As a result, several indirect assays have been used as a measure of transpeptidase activity.

The most commonly used assay to screen for transpeptidase

* Corresponding author. Mailing address: AstraZeneca India Pvt. Ltd., Hebbal, Bellary Rd., Bangalore 560 024, India. Phone: 91-80-362-1212. Fax: 91-80-362 1214. E-mail: sunita.desousa@astrazeneca.com.
† Present address: Plexxikon, Berkeley, CA 94710.
‡ Present address: 12/7 New Dhamu Nagar, Coimbatore 641 037, India.
§ Present address: Medical College of Ohio, Toledo, Ohio.

inhibitors monitors binding of a labeled β-lactam, e.g., penicillin, to the PBPs and competition of this binding by a test compound (22). Cleavage of a chromogenic β-lactam, e.g., nitrocefin, on binding to the PBP can also be used (21). Both these assays are amenable to high-throughput screening but measure binding to an inhibitor rather than enzyme activity. Other assays for the transpeptidase measure UDP–*N*-acetylglucosamine (UDP-GlcNAc)-dependent cleavage of the terminal (fifth) amino acid of UDP-MurNAcpp or cleavage by the PBP of synthetic substrates that mimic the stem peptide, which can be monitored colorimetrically (1, 16).

A high-throughput screen to detect inhibitors of five enzymes involved in peptidoglycan synthesis was reported previously (8). It is a true enzyme assay for both the transglycosylase and transpeptidase and can pick up inhibitors of both the transglycosylase and transpeptidase; but it cannot distinguish these from inhibitors of MraY, MurG, or lipid pyrophosphorylase (8). In optimizing the activity of an inhibitor it is important to know which of the five enzymes is being inhibited. Here we report on a high-throughput method that can be used to select inhibitors of the activities of the PBPs, i.e., the transglycosylase or the transpeptidase activities, or both, from those of the other three enzymes. Peptidoglycan synthesis was allowed to occur in *E. coli* membranes, and at the end of the reaction the product was captured by wheat germ agglutinin (WGA)-coated scintillation proximity assay (SPA) (WGA-SPA) beads in the presence or absence of detergent. The differential effects of the two capture methods on inhibitors of the transglycosylase and transpeptidase (moenomycin and penicillin, respectively) allow these inhibitors to be distinguished from inhibitors of the other enzymes. In another set of assays, the reaction products captured with WGA-SPA beads and type A polyethyleneimine (PEI)-coated WGA-SPA (PEI-WGA-SPA) beads (in the presence of detergents) were compared. Inhibitors of all five enzymes inhibited product capture with the WGA-SPA beads and could be selected from among the compounds with no effect. The β-lactams, inhibitors of the transpeptidase, alone showed insignificant inhibition when product capture was with the type A PEI-WGA beads, thus providing a means to select for transpeptidase inhibitors.

(Part of this work was presented at the 42nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, Calif., 2002 [B. Chandrakala et al., Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., posters F-718 and F720, 2002].)

MATERIALS AND METHODS

Materials. WGA-SPA beads (polyvinyltoluidene [PVT] beads; RPNQ0001) or type A PEI-WGA-SPA beads (PVT beads; RPNQ0003) were from Amersham International plc (Little Chalfont, United Kingdom). UDP-[^{3}H]GlcNAc was from Dupont, NEN Research Products (Boston, Mass.). Most chemicals were from Sigma Chemical Co. (St. Louis, Mo.). Flavomycin (moenomycin) was a gift from Hoechst (Bombay, India). Antibiotic medium 3 was from Difco Laboratories (Detroit, Mich.). Chromatography materials were from Bio-Rad Laboratories (Richmond, Calif.) or Whatman, Inc. (Clifton, N.J.). Mutants of *E. coli* AMA1004 with mutations in the genes for PBP 1b (AMA1004 *ponB*::Spc^r) or PBP 1a (AMA1004 Δ*ponA*) were generated in-house as described earlier (31); the former was grown in Luria-Bertani broth containing 50 μg of spectinomycin per ml.

Substrates. UDP-MurNAcpp was purified from *Bacillus cereus* 6A1 as described earlier (8, 12). Briefly, a hot water extract of the cells was purified by gel filtration followed by ion-exchange chromatography. The concentration of the UDP-MurNAcpp was estimated by determination of its absorbance at 262 nm by using a molar extinction coefficient of 10,000.

Enzyme preparation. Membranes were prepared from *E. coli* AMA1004 or the mutants as described earlier (8, 12). Briefly, the cells (in 50 mM Tris-HCl [pH 7.5], 0.1 mM $MgCl_2$) were lysed in a French pressure cell. The supernatant obtained after low-speed centrifugation was centrifuged at 150,000 × *g*, and the pellet was washed once and used as the enzyme source. The quality of each membrane batch was monitored by measuring the amount of peptidoglycan synthesized by different quantities of protein (8) as well as by determination of the counts per minute obtained in the blank reaction (see below).

Enzyme assay for peptidoglycan synthesis. The enzyme assay for peptidoglycan synthesis was performed as described earlier (8) in flexible 96-well plates (1450-401; Wallac, Turku, Finland). Membranes (with 4 μg of protein) were incubated for 90 min at 37°C with 75 μM UDP-MurNAcpp and 2.5 μM UDP-[^{3}H]GlcNAc (0.1 μCi) in a buffer consisting of 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 4% dimethyl sulfoxide in a final volume of 25 μl. Reactions were carried out in triplicate. The enzyme reaction was stopped by addition of 5 μl of 90 mM EDTA. Reactions were analyzed after addition of 170 μl of SPA beads. Radioactivity was measured directly in a Microbeta Trilux liquid scintillation and luminescence counter 3 to 16 h after addition of the beads.

A reaction without the first sugar nucleotide (UDP-MurNAcpp) was run in parallel. This was treated as a blank, and for each type of capture condition, the counts per minute obtained in this reaction was subtracted from that obtained in reactions containing both sugar precursors (complete or 100% reaction) as a measure of peptidoglycan synthesis. To determine the effects of the inhibitors, the activity was first calculated (by subtracting the reading for the blank well from the reading obtained for the well containing an inhibitor), and then percent inhibition was calculated as 100 − [(activity of inhibitor well × 100)/activity of 100% reaction]. For each set of capture conditions, blank and 100% control wells were set up, and these were used to calculate the percent inhibition by the test compounds for that particular capture condition.

Radioactive GlcNAc was incorporated into peptidoglycan, and the quantity of peptidoglycan formed in the reaction mixture was measured as the counts per minute detected by WGA-SPA in the presence of detergent, e.g., Triton X-100. We have shown earlier that this type of SPA reflects the quantity of GlcNAc incorporated into peptidoglycan and that it can replace analysis by paper chromatography (8). For the SPA it is difficult to determine the counting efficiency, so the results are represented as counts per minute. When the purity of the inhibitor is not defined, concentrations are expressed in units other than micromolar. For analysis by paper chromatography, the reaction was stopped by the addition of EDTA, and samples were spotted onto Whatman 3MM filter paper, which was chromatographed in isobutyric acid–1 M ammonia (5:3; vol/vol); peptidoglycan runs at the origin, and lipids I and II run with R_fs of ~0.9.

Assays for selection of inhibitors of the transglycosylase and/or transpeptidase. For selection of inhibitors of the activities of both the transglycosylase and transpeptidase of the PBPs, two sets of enzyme reactions were run in parallel and the products were captured with SPA beads either in the absence or the presence of detergent. The WGA-SPA beads (500 μg; RPNQ0001; Amersham) were added as a suspension in Tris buffer, such that the final concentration (in 200 μl) was 100 mM Tris-HCl (pH 7.5). In wells in which detergent was used at the capture step, the detergent was added along with the bead suspension so that the final concentration of Triton X-100 (in 200 μl) was 0.05%; when Sarkosyl was used, the final concentration was 0.2%.

Assays for selection of transpeptidase inhibitors. For selection of inhibitors of the transpeptidase, two sets of enzyme reactions were run in parallel and captured with either WGA-SPA beads in the presence of detergent or type A PEI-WGA beads in the presence of detergent. The enzyme reaction conditions for this set of experiments (see Fig. 3 and Tables 3 and 4) were similar to those described above, except for the following changes. The buffer used was 50 mM HEPES–ammonia (pH 7.5) with 15 μM UDP-MurNAcpp and 2.5 μM UDP-[^{3}H]GlcNAc (0.15 μCi). In these sets of experiments the reactions were carried out in duplicate.

The WGA-SPA beads (500 μg/well) were added as a suspension in Sarkosyl-HEPES buffer such that the final concentrations (in 200 μl) were 0.2% Sarkosyl and 50 mM HEPES-ammonia (pH 7.5). The type A PEI-WGA beads (500 μg/well; RPNQ0003; Amersham) were added as a suspension in Triton X-100–HEPES buffer so that the final concentrations (in 200 μl) were 0.05% Triton X-100 and 50 mM HEPES-ammonia (pH 7.5).

RESULTS

Selection of inhibitors of the PBPs. (i) Inhibitors of the MraY, MurG, and lipid pyrophosphorylase. In the initial assay for the measurement of peptidoglycan synthesis (8), Triton

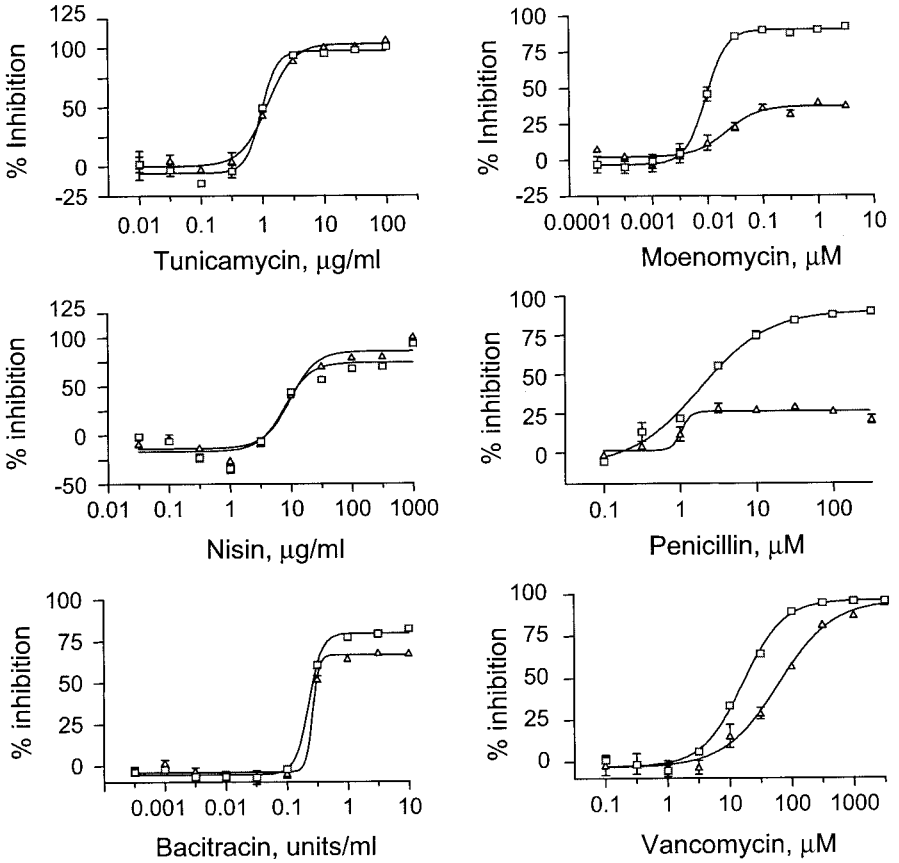


FIG. 1. Effects of inhibitors of peptidoglycan synthesis on reaction sets in which the products were captured by WGA-SPA beads in the presence of Triton X-100 (squares) or in the absence of the detergent (triangles). Tests were performed with inhibitors of the MraY (tunicamycin), MurG (nisin), lipid pyrophosphorylase (bacitracin), transglycosylase (moenomycin), and transpeptidase (penicillin G) activities of the PBPs and vancomycin. The activities obtained for tunicamycin were as follows: for capture by WGA-SPA beads, 1,552 cpm for the complete reaction and 455 cpm for the blank reaction; for capture by WGA-SPA beads in the presence of Triton X-100, 1,039 cpm for the complete reaction and 138 cpm for the blank reaction. The values were of the same range for the other inhibitors.

X-100 was included along with the WGA-SPA beads at the capture step to reduce the background (reactions with no UDP-MurNAcpp) signal in the assay. To determine if the presence of Triton X-100 was essential, the effects of inhibitors of peptidoglycan synthesis were studied in two parallel sets of reactions, in one set of which the products were captured by WGA-SPA beads in the presence of Triton X-100 and in the other set of which the products were captured by the same beads in the absence of the detergent (Fig. 1). The only difference in the two sets of reactions is the presence of detergent at the step of analysis of the product formed, i.e., after the enzyme reaction had been stopped by EDTA (see Fig. 4). For each set of reactions, the percent inhibition by a test compound was calculated by comparison of the activities in the wells with the test compound with the activities in the wells with no inhibitor in which the products were captured under identical conditions.

For tunicamycin, an inhibitor of the MraY (6), the same effect was seen in both sets of analyses, i.e., whether or not Triton X-100 was present along with the WGA-SPA beads at the time of capture. The 50% inhibitory concentrations (IC_{50}s) were very similar under the two capture conditions, and with the highest concentration of inhibitor tested, the level of inhibition was >90%. The results observed with nisin, an inhibitor of the MurG, and bacitracin, an inhibitor of the lipid pyrophosphorylase, were similar to those observed with tunicamycin (Fig. 1).

(ii) Inhibitors of the transglycosylase or transpeptidase activities of PBPs. The effect of moenomycin, an inhibitor of the transglycosylase, on peptidoglycan synthesis was very different from those of the inhibitors described above (Fig. 1). Potent inhibition was seen in the reaction set in which the products were captured by WGA-SPA beads in the presence of Triton X-100 (IC_{50}, ~10 nM). However, insignificant inhibition was seen when the product was captured in the absence of detergent, with the maximum inhibition rarely reaching 50% (IC_{50}, >10 μM). Increasing the concentration of moenomycin did not significantly increase the inhibition.

Penicillin G and other β-lactam antibiotics, which are inhibitors of the transpeptidase, also showed effects similar to that of moenomycin. When the reaction products were captured by WGA-SPA beads in the presence of Triton X-100, the IC_{50} of penicillin was ~3 μM. However, in the set in which the products were captured in the absence of detergent, insignificant

TABLE 1. Comparison of the IC_{50}s of inhibitors of peptidoglycan synthesis when reaction products were captured by WGA-SPA beads in the presence or the absence of Triton X-100

Drug (concn units)	Enzyme target[a]	IC_{50}		Maximum % inhibition[b] (no detergent)
		With Triton X-100	Without detergent[c]	
Tunicamycin (μg/ml)	MraY	1	1.2	100
Nisin (μg/ml)	MurG	13	13	80
Vancomycin (μM)	MraY, MurG, TG, TP	18	73	80
Ristocetin (μg/ml)	MraY, MurG, TG, TP	6	82	50
Bacitracin (U/ml)	Lipid pyrophosphorylase	0.3	0.3	68
Moenomycin (μM)	TG	0.01	>3	35
Penicillin G (μM)	TP	3	>300	30
Ampicillin (μM)	TP	10	>1,000	40
Meropenem (μM)	TP	87	>3,000	0

[a] TG, transglycosylase; TP, transpeptidase.
[b] The maximum inhibition observed for the range of drug concentrations tested.
[c] The inhibition in the absence of detergent varied somewhat between experiments but seldom reached 50% inhibition for either moenomycin or the β-lactams tested.

inhibition (usually far less than 50%) was seen. Similar effects were observed with meropenem and other β-lactam antibiotics (Table 1).

The β-lactam needs to be present at the start of the enzyme reaction for inhibition to be observed (8). If it was added after the reaction had been stopped by EDTA, no inhibition was observed, even when the reaction products were captured with WGA-SPA beads in the presence of detergent; under these conditions the β-lactam should bind covalently to the PBP but would not be able to inhibit the cross-linking of peptidoglycan since the reaction had been stopped. We have shown earlier that the reaction that occurs during incubation of membranes with the two sugar precursors is enzyme mediated and results in synthesis of peptidoglycan (8). This indicates that these are true enzyme assays for the transpeptidase, and mere binding of the β-lactam to the PBPs in the membranes is insufficient to cause inhibition.

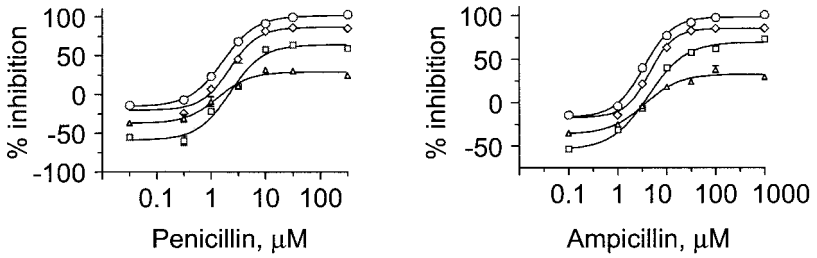
Both moenomycin and penicillin are inhibitors of the PBPs, and by this method inhibitors of the PBPs can be distinguished from those of the MraY, MurG, or lipid pyrophosphorylase (see Fig. 4).

(iii) Vancomycin and ristocetin. Like the other inhibitors described, vancomycin showed inhibitory activity (IC_{50}, ~20 μM) when the reaction products were captured by WGA-SPA beads in the presence of detergent (Fig. 1). In the set of reactions in which the products were captured in the absence of Triton X-100, unlike inhibitors of the PBPs, significant inhibition was observed, with the maximum inhibition reaching >90%. However, the IC_{50} of vancomycin under these conditions (~75 μM) was often considerably higher than that obtained in the set of reactions in which the products were captured by WGA-SPA beads in the presence of detergent. In this respect, vancomycin is different from nisin, tunicamycin, and bacitracin, the IC_{50}s of which were nearly identical under the two conditions. When the two sets of data are plotted on the same graph, the two lines diverge initially and then converge for the higher concentrations of inhibitor, in contrast to tunicamycin and nisin, for which the two lines were parallel and coincident, indicating nearly identical IC_{50}s (Fig. 1). The effect of vancomycin is midway between the effects seen with these inhibitors and the patterns observed for the inhibitors of the PBPs.

Vancomycin is thought of as an inhibitor of the transglycosylase or transpeptidase, but in a cell-free system it inhibits the MraY and MurG as well. The graphs are probably a reflection of this and suggest a difference in the IC_{50} for the translgycosylase-transpeptidase versus that for the MraY or MurG. For example, at low concentrations (~10 μM), at which vancomycin presumably inhibits the translgycosylase or transpeptidase, it shows poor inhibition in reactions in which the products are captured in the absence of Triton X-100 (as do inhibitors of these two enzymes), whereas at high concentrations (1 mM), it inhibits reactions in which the products are captured in the absence of detergent, as would an inhibitor of the MraY or MurG (Fig. 1). A similar effect was observed with ristocetin, which is also a glycopeptide antibiotic with a mode of action similar to that of vancomycin. Both antibiotics bind to the terminal part of the stem peptide attached to the muramyl sugar, thus inhibiting all the enzymes when this is part of the substrate.

(iv) Effects of other detergents on capture by WGA-SPA beads. Many of the β-lactams showed maximum inhibition of <90% in reactions in which the products were captured in the presence of Triton X-100, even though the inhibitor concentration was increased to 3 mM. This was not observed with the other inhibitors tested and appears to be peculiar to the β-lactams. Increasing the Triton X-100 concentration to 1% did not result in increased inhibition even at the highest concentration of β-lactam. To check whether this is specific to Triton X-100, other detergents were tested for their effects on capture by the WGA-SPA beads. With Sarkosyl at a concentration of 0.2% and octyl-β-glucoside at a concentration of 1%, the maximum inhibition observed was much higher (~100%) than that obtained with Triton X-100 (Fig. 2), and the resulting IC_{50}s were three to four times lower than those obtained when the products were captured with Triton X-100. Hence, in later experiments Sarkosyl was used instead of Triton X-100.

(v) What is bound to the WGA-SPA bead in the presence or absence of detergent? To analyze what was captured by the bead, two sets of reactions were performed in parallel and the products were captured by either WGA-SPA beads in buffer or the same beads in the presence of 0.2% Sarkosyl. Besides the complete and the blank reactions, enzyme reactions were per-



Detergent	Penicillin IC_{50}	Ampicillin IC_{50}
None	> 300	>1000
Sarkosyl	2	4
Octyl-β-glucoside	3	7
TritonX-100	9	18

FIG. 2. Effects of penicillin G and ampicillin when reaction products were captured by WGA-SPA beads under different conditions: no detergent (triangles), 0.05% Triton X-100 (squares), 0.2% Sarkosyl (circles), and 1% octyl-β-glucoside (diamonds). The concentration of UDP-MurNAcpp in this experiment was 15 μM, and the buffer used was 50 mM HEPES-ammonia (pH 7.5).

formed in the presence of 100 μM penicillin G and 1 μM moenomycin (Table 2).

A third set of reactions was analyzed by paper chromatography. In the complete reaction the only product that formed (in comparison with the blank) was peptidoglycan, at the origin of the paper chromatogram, and insignificant radiolabel was incorporated into lipid II (8). The same was observed for the reaction in the presence of penicillin G. However, in this case we would expect that the product would be un-cross-linked peptidoglycan, whereas in the former it would be cross-linked peptidoglycan (16); the paper chromatogram could not distinguish between nascent and cross-linked peptidoglycan. In the presence of moenomycin the only significant radiolabeled product was lipid II (R_f, ~0.9); no peptidogycan was formed (data not shown) (8).

Penicillin G and moenomycin showed poor inhibition in the reaction set in which the products were captured by WGA-SPA beads in the absence of detergent (Table 2). After the activity was measuring by SPA, the beads were twice washed with buffer and the radiolabel associated with them was counted by addition of scintillation fluid. Significant radiolabel was associated with the beads in the complete reaction as well as those in the reactions with moenomycin; radiolabel was also associated with the beads in the reactions with penicillin G, although the level was lower than those in the other two cases. This suggests that the WGA-SPA bead captures the products observed in these three reactions, cross-linked peptidoglyan, un-cross-linked peptidoglycan, and lipid II (Table 2), and explains the poor inhibition by penicillin and moenomycin.

However, the results were clearly different for the reaction set in which the products were captured by WGA-SPA beads in the presence of 0.2% Sarkosyl. Penicillin G and moenomycin showed >90% inhibition. After capture the beads were washed in buffer with Sarkosyl and the radiolabel associated with them was counted by addition of scintillation fluid. Only in the complete reaction, in which the sole product is peptidoglycan (which is expected to be cross-linked), was there significant radioactivity on the beads; in enzyme reactions performed in

TABLE 2. Radiolabel associated with WGA-SPA beads when reaction products were captured in the presence or the absence of Sarkosyl

Inhibitor	Radiolabel (cpm) by capture with:			
	WGA-SPA beads		WGA-SPA beads with Sarkosyl	
	Activity by SPA[a]	Radiolabel on washed beads[b]	Activity by SPA	Radiolabel on washed beads[b]
None	8,091	7,189	34,215	8,061
Penicillin G (100 μM)	3,965 (51)	2,098	1,989 (94)	307
Moenomycin (1 μM)	7,964 (2)	8,361	347 (99)	72

[a] The counts on the plates were determined 17 h after bead addition. The numbers in parenthesis are percent inhibition compared to that in the reaction with no inhibitor. The values reported as activity are those after subtraction of the values for the blank (i.e., the reaction with no UDP-MurNAcpp); the blank values were as follows: for WGA-SPA bead capture set, 3,043 cpm by SPA and 861 cpm for the radiolabel remaining on the washed bead; for the WGA-SPA–Sarkosyl capture set, 1,997 cpm by SPA and 82 cpm for the radiolabel remaining on the washed bead.

[b] Following reading of the results obtained by SPA, the beads were centrifuged, washed twice with 100 μl of 50 mM HEPES-ammonia buffer (pH 7.5) without detergent or with 0.2% Sarkosyl, and resuspended in 100 μl of the same buffer; and the counts in 10 μl were determined after addition of 200 μl scintillation fluid. In this experiment the following conditions were different: the enzyme reaction was performed with 1.5 μCi of UDP-[^{3}H]GlcNAc, 15 μM UDP-MurNAcpp, the buffer used was 50 mM HEPES-ammonia (pH7.5), and the final volume after bead addition was 100 μl.

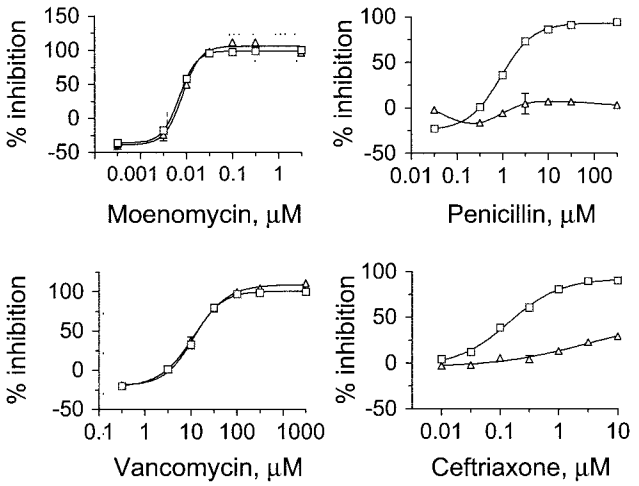


FIG. 3. Effects of inhibitors (IC_{50}s) of peptidoglycan synthesis on capture of the reaction products by the WGA-SPA beads (triangles) and type A PEI-WGA-SPA beads (squares). Enzyme reactions were performed in parallel; after the reaction was stopped, either WGA-SPA beads (squares) or type A PEI-WGA-SPA beads (triangles), along with detergent, were added to the set of reactions. Tests were performed with inhibitors of the transglycosylase (moenomycin); an inhibitor of the MraY, MurG, transglycosylase, and transpeptidase (vancomycin); and β-lactams, inhibitors of the transpeptidase (penicillin and ceftriaxone). The values obtained in the experiment with moenomycin were as follows: for capture by WGA-SPA beads, 284 cpm for the reaction with the blank and 2,727 cpm for the complete reaction; for type A PEI-WGA-SPA beads, 1,001 cpm for the blank reaction and 2,771 cpm for the complete reaction. The values were of a similar range for the other inhibitors.

the presence of penicillin G or moenomycin, insignificant radioactivity was associated with the beads. This suggests that in the presence of detergent the WGA-SPA beads capture only cross-linked peptidoglycan and not nascent peptidoglycan or lipid II. Unfortunately, we were unable to accumulate sufficient radiolabeled product on the bead to allow elution of the material and analysis by paper chromatography. The elution methods that we tried were not efficient, and the paper quenched the radioactivity (giving only 10% of the efficiency obtained by counting the radioactivity directly in scintillation fluid), resulting in no signal on the paper chromatogram.

Assay for selection of transpeptidase inhibitors. Two sets of peptidoglycan-synthesizing enzyme reactions were run in parallel in a microtiter plate, and after the enzyme reaction was stopped with EDTA, the products were captured either by WGA-SPA beads plus detergent (8) or by type A PEI-WGA-SPA beads in the presence of detergent. Inhibitors of the MraY (tunicamycin), MurG (nisin), lipid pyrophosphorylase (bacitracin), and transglycosylase (moenomycin) showed inhibitory activity when the reaction products were captured either by the WGA-SPA beads or by the type A PEI-WGA-SPA beads, giving near identical IC_{50}s under both conditions (Fig. 3; Table 3). Vancomycin also showed inhibitory activity in the reactions in which the products were captured with both the WGA-SPA beads and the type A PEI-WGA-SPA beads.

However, the effects of the β-lactams were distinctly different from those of these inhibitors. Penicillin G showed inhibitory activity when the product was captured by the WGA-SPA beads, as reported earlier (8), but it had no effect whatsoever when the product was captured by the type A PEI-WGA-SPA beads. The same effect was seen with other β-lactams, e.g., ampicillin, ceftriaxone, cefazolin, aztreonam, and meropenem, all of which showed inhibitory activity when the product of the reaction was captured by the WGA-SPA beads but which showed insignificant inhibitory activity when the product was captured by using the type A PEI-WGA SPA beads (Fig. 3; Table 3). Since it is well accepted that the β-lactams are the inhibitors of the transpeptidase (16), we expect other transpeptidase inhibitors to show the same effect. Thus, those compounds that show inhibitory activity when the reaction prod-

TABLE 3. Comparison of IC_{50}s of compounds in enzyme reactions in which products were captured by WGA or type A PEI-WGA-SPA beads[a]

Inhibitor (concn units)	Enzyme inhibited[b]	IC_{50}	
		WGA-SPA beads	Type A PEI-WGA-SPA beads
Tunicamycin (μg/ml)	MraY	0.3	0.2
Nisin (μg/ml)	MurG	7	11
Moenomycin (μM)	TG	0.01	0.01
Vancomycin (μM)	MraY, MurG, TG, TP	14	14
Ristocetin (μg/ml)	MraY, MurG, TG, TP	5	4
Bacitracin (U/ml)	Lipid pyrophosphorylase	0.4	0.4
Penicillin G (μM)	TP	2	>300
Ampicillin (μM)	TP	4	>1,000
Amoxicillin (μM)	TP	8	>1,000
Meropenem (μM)	TP	17	>1,000
Aztreonam (μM)	TP	11	>1,000
Cefazolin (μM)	TP	15	>1,000
Cephapirin (μM)	TP	14	>1,000
Cefaclor (μM)	TP	11	>1,000
Ceftriaxone (μM)	TP	0.2	>1,000
Cefuroxime (μM)	TP	4	>1,000
Cephadroxil (μM)	TP	485	>1,000
Cephalexin (μM)	TP	863	>1,000
Cephradine (μM)	TP	953	>1,000

[a] Enzyme reactions were performed in parallel, and the products were analyzed by addition of either WGA-SPA beads or type A PEI-WGA-SPA beads, both in the presence of detergent.
[b] TG, transglycosylase; TP, transpeptidase.

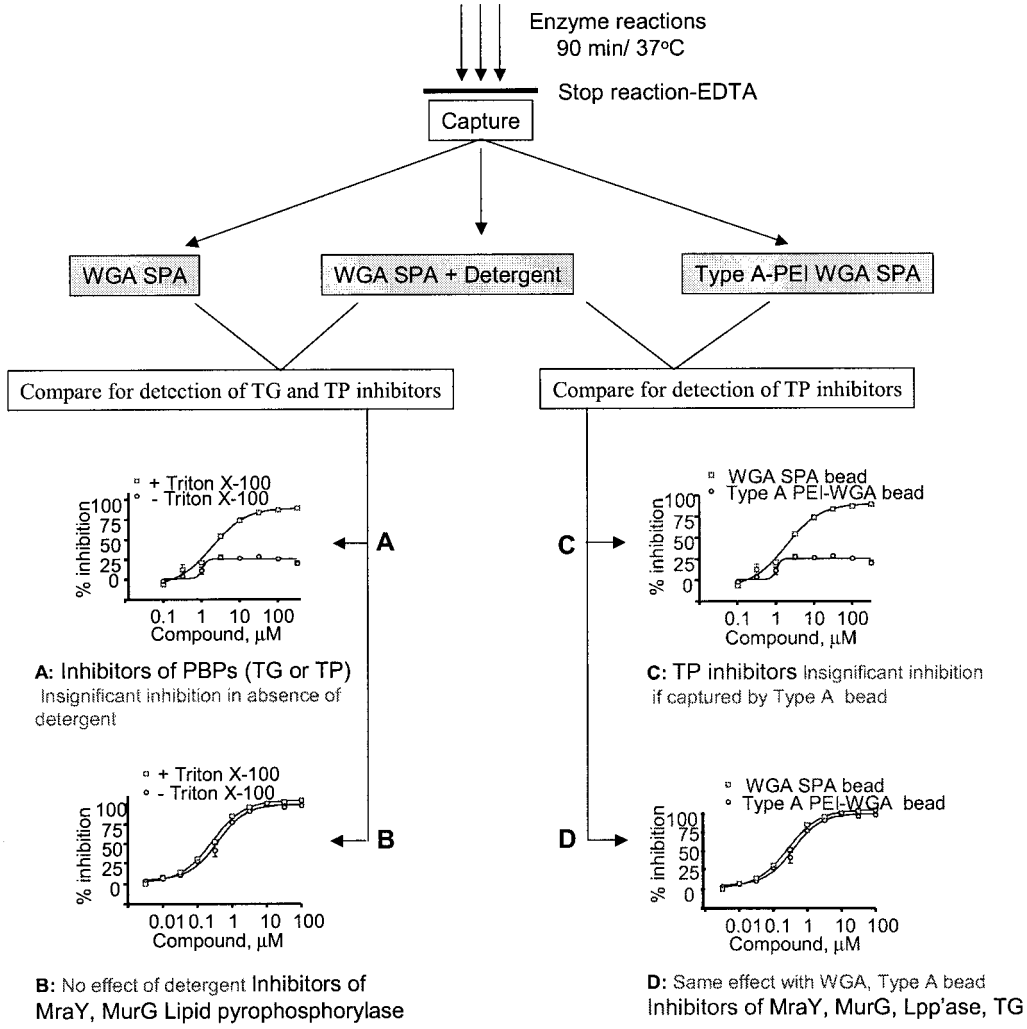


FIG. 4. Schematic of principle for selection of inhibitors of the transglycosylase (TG) or transpeptidase (TP) activities of PBPs. For determination of inhibitors of the activity of either enzyme of the PBP, WGA-SPA beads were added after termination of the enzyme reaction with EDTA. In one set detergent was added along with the WGA-SPA beads (squares). Inhibitors of the PBPs show insignificant inhibition when the reaction products are captured by WGA-SPA beads in the absence of detergent (circles). For detection of transpeptidase inhibitors, WGA-SPA beads were added to one set of reactions and type A PEI-WGA SPA beads were added to the other set, both in the presence of detergent. Inhibitors of the MraY, MurG, transglycosylase, and lipid pyrophosphorylase (Lpp'ase) had inhibitory activity when the reaction products were captured with either WGA-SPA beads (squares) or type A PEI-WGA SPA beads (circles). Inhibitors of transpeptidase had inhibitory activity only when the reaction product was captured with WGA-SPA beads (squares) and showed insignificant inhibitory activity when the reaction product was captured with type A PEI-WGA-SPA beads (circles).

ucts are captured with WGA-SPA beads (plus detergent) but not with the type A PEI-WGA-SPA beads can be selected as transpeptidase inhibitors (Fig. 4).

In some cases, particularly with the β-lactams, negative inhibition, i.e., activation, was seen at low concentrations of the inhibitor; this has been observed before (8, 15). It could reflect β-lactam inhibition of carboxypeptidases and endopeptidases, which would otherwise cleave the peptide of UDP-MurNAcpp, or genuine stimulation of the PBP by the inhibitor.

PBP 1b activity is being measured in the assay. The *E. coli* cell has many PBPs, and the observation made above raised the question of which transpeptidase activity was being measured in the enzyme reaction. One indication would be the effects of transpeptidase inhibitors that are specific to one of the PBPs.

Aztreonam has a higher affinity for PBP 3 than for PBP 1b, whereas penicillin and ampicillin bind to most PBPs in the cell; all three showed inhibitory activity when the reaction products were captured with WGA-SPA beads. Cephalexin and cephradine bind primarily to PBP 1a and PBP 3 of *E. coli* (10), and both showed very poor inhibitory activities when the reaction products were captured with the WGA-SPA beads (Table 1). On the other hand, all the β-lactams that showed inhibitory activities when the products were captured with the WGA-SPA beads bind to PBP 1b (10). This suggests that the major transpeptidase activity being measured in this assay is that of PBP 1b.

Another way to determine which PBP is being measured in this assay would be to use membranes from an *E. coli* strain

TABLE 4. Effects of different agents on capture of peptidoglycan by WGA-SPA or type A PEI-WGA-SPA beads[a]

Capture condition	WGA-SPA beads		Type A PEI-WGA-SPA beads	
	Activity (cpm)	% Inhibition	Activity (cpm)	% Inhibition
No addition	1,309	0	954	0
GlcNAc (0.5 M)	418	68	566	41
KCl (0.5 M)	1,755	−34	414	57
KCl (2 M)	1,569	−20	2,988	−213
Triton X-100 (0.05%)	1,796	−37	2,674	−180
Sarkosyl (0.2%)	1,661	−27	248	74

[a] The agents were added after the enzyme reaction was stopped and before addition of the SPA beads. For the WGA-SPA beads the blank was 790 cpm, and for the type A PEI-WGA-SPA beads the blank was 504 cpm. Percent inhibition was calculated with respect to the activity when beads alone were added.

with a mutation for one or more PBPs. The results of the routine assay with membranes from *E. coli* AMA1004 were compared with those of assays in which the membrane used was from two PBP mutants with the same genetic background as AMA1004; the total amount of protein used in each assay was the same in all three cases. The quantity of peptidoglycan synthesized by 4 μg of membrane from the strain with a mutation for PBP 1a was similar (100%) to that synthesized by 4 μg of membrane from the wild-type *E. coli* strain. However, when the membrane from a strain with a mutation for PBP 1b was used, an insignificant quantity of peptidoglycan was synthesized (6% of that produced by the wild type by using 4 μg of protein; data not shown). Even when the quantity of membrane protein from the mutant with the PBP 1b mutation was increased to 16 μg, very little peptidoglycan was synthesized (20% of that synthesized by 4 μg of the wild-type membrane), indicating that the activity of PBP 1a is much less than that of PBP 1b under these assay conditions. This indicates that the major PBP being measured under these conditions is PBP 1b, although one cannot distinguish between the transglycosylase or transpeptidase activity of PBP 1b that is responsible for this, since both enzymes reside on the same polypeptide. This is in keeping with an earlier report (12) that ~96% of the activity of the wild-type membrane measured in vitro is contributed by PBP 1b when peptidoglycan synthesis is monitored by paper chromatography. We expect that if the reaction conditions are altered the activity of PBP 1a could be monitored under assay conditions similar to those described here.

Mechanism of capture by SPA beads. To understand how the two types of SPA beads capture peptidoglycan, the products of a complete reaction were captured by either bead type in the presence of agents expected to compete with the product for binding to the bead. Chitobiose, a dimer of GlcNAc, partially inhibited the capture of peptidoglycan by the WGA-SPA beads (~37 and ~80% inhibition by 1 and 10 mM chitobiose, respectively). GlcNAc was less effective in inhibiting capture (~70% inhibition by 500 mM GlcNAc), whereas potassium chloride (0.5 or 2 M) had no effect (Table 4). The greater effectiveness of chitobiose in preventing capture is in keeping with a report (2) that the binding of chitobiose to WGA is 600 times more potent than that of GlcNAc. The WGA-SPA beads seem to bring the labeled peptidoglycan into their proximity primarily by binding to GlcNAc residues.

Partial inhibition of capture by the type A PEI-WGA-SPA beads was observed with GlcNAc (~40%) as well as with 0.5 M potassium chloride and 0.2% Sarkosyl, an ionic detergent. However, 2 M KCl increased the quantity of product captured. This suggests that capture by this bead type is more complex and involves binding to GlcNAc residues but that ionic and hydrophobic interactions also have a role. The two bead types capture products by different mechanisms, so it is not surprising if they capture different products.

DISCUSSION

All five enzymes as well as the lipid carrier undecaprenol phosphate, which is involved in the late stages of peptidoglycan synthesis, are membrane associated. Thus, by incubating membranes with the two UDP-linked sugar precursors, UDP-MurNAcpp and UDP-GlcNAc, this part of the peptidoglycan synthetic pathway can be reproduced in a cell-free system (3, 15, 17). The five enzymes work sequentially to incorporate the sugars into cross-linked peptidoglycan, and this can be easily monitored if one sugar precursor is radiolabeled.

It was reported earlier that SPA technology (9) can be used to monitor the synthesis of peptidoglycan by these five enzymes (8) and can replace the traditional analysis by paper chromatography (3). WGA-SPA beads are added at the end of the enzyme reaction, and the radioactivity is measured in the same plate, resulting in a simple assay. WGA binds to GlcNAc, and presumably by binding to these residues in components of the cell wall, radioactive peptidoglycan (but not UDP-[^{3}H]GlcNAc) is brought into the proximity of the scintillant on the SPA bead and is counted. WGA-SPA beads are frequently used in mammalian receptor binding assays, in which a receptor-bound radioactive ligand is brought into the proximity of the bead scintillant by binding of the bead to GlcNAc residues on membrane proteins (9, 20).

The assay reported earlier can detect inhibitors of peptidoglycan synthesis, but it cannot determine which of five enzymes is being inhibited (8). While inhibitors of the MraY and MurG can be detected by assaying the individual enzymes, assay of the transglycosylase and transpeptidase activities of PBPs is notoriously tedious. Here we describe a modification of the earlier assay that can easily distinguish inhibitors of the transglycosylase and/or transpeptidase from those of the MraY, MurG, and lipid pyrophosphorylase. Both tests rely on comparison of two sets of enzyme reactions that are set up under identical conditions; thus, the products in both reaction sets would be the same. The only difference is in the analysis of the products formed after the termination of the enzyme reaction. Thus, the SPA beads must capture different products under the three different conditions.

Since synthesis of cross-linked peptidoglycan in this assay is a consequence of the serial actions of five enzymes, different intermediates would accumulate on inhibition of any one of the enzymes (Fig. 5). Inhibition of the first enzyme, MraY, would result in no product. If MurG were inhibited, the product would be lipid I, which would not be radioactive, since the radiolabel used is UDP-GlcNAc, the MurG substrate. If the lipid pyrophosphorylase were inhibited, there should be only one round of peptidoglycan synthesis (since the lipid carrier cannot be recycled), so an insignificant quantity of radiolabeled

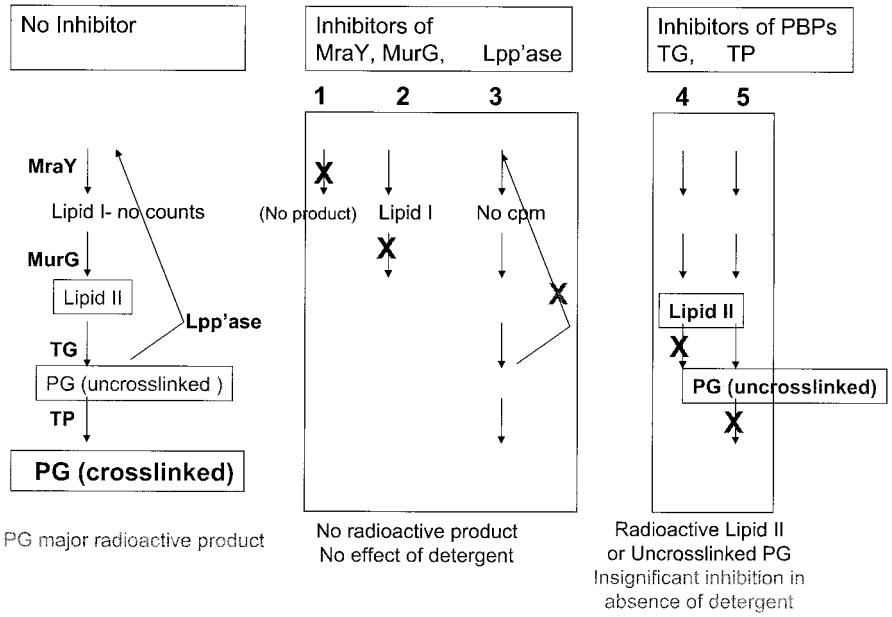


FIG. 5. Schematic of membrane-associated steps of peptidoglycan synthesis showing the products expected in the presence of inhibitors of each of the five enzymes. The enzymes mediating the reactions are shown on the left; radioactive products are boxed. In the absence of any inhibitor cross-linked peptidoglycan is the major radioactive product. In the presence of inhibitors of the MraY, MurG, or lipid pyrophosphorylase, no radioactive product would be formed. In the presence of transglycosylase inhibitors, lipid II would be the major radioactive product, and in the presence of transpeptidase inhibitors, un-cross-linked peptidoglycan would be the major radioactive products. Abbreviations: PG, peptidoglycan; TG, transglycosylase; TP, transpeptidase; Lpp'ase, lipid pyrophosphorylase.

peptidoglycan would be formed. Hence, in assays with inhibitors of these three enzymes, since practically no radioactive product is formed, irrespective of what moiety is captured by the SPA bead, there would be no difference in the results when any two capture conditions were compared. It is therefore not surprising that there was no difference in the level of inhibition (IC_{50}) when the reaction products were captured by WGA-SPA beads in the presence or absence of detergent or by the type A PEI-WGA-SPA beads. This explains the results observed for tunicamycin, nisin, and bacitracin when the reaction products were captured under the three conditions described above.

In the absence of any inhibitor the major radioactive product is peptidoglycan. In contrast, when the transglycosylase is inhibited, for example, by moenomycin, radiolabeled lipid II accumulates, as observed by paper chromatography. The results presented here can be explained if in the presence of detergent only cross-linked peptidoglycan and not lipid II is captured, hence the inhibition by moenomycin. We speculate that the lipid being anchored to the cytoplasmic membrane would be solubilized or removed from the proximity of the SPA bead by the detergent. In the absence of detergent, if both lipid II and peptidoglycan were captured by the WGA-SPA beads, moenomycin would show insignificant inhibitory activity; this is supported by the data in Table 2.

Inhibition of the transpeptidase would result in radioactive nascent peptidoglycan, i.e., material that is not cross-linked. It is difficult to imagine why this is not captured by WGA-SPA beads in the presence of detergent, except that it has been proposed that newly formed peptidoglycan is attached to preexisting peptidoglycan by the transpeptidation reaction (14, 28). In this case nascent peptidoglycan would still be attached to the inner membrane, and this would behave very much like lipid II. This is supported by the data in Table 2, although it appears that the capture of un-cross-linked peptidoglycan in the absence of detergent is not very efficient. Our attempts to elute the material from the bead with detergent were unsuccessful.

Our conclusion is that in the presence of detergent only cross-linked peptidoglycan is brought into the proximity of the WGA-SPA bead, suggesting that the bead binds to preexisting peptidoglycan or a component of the outer membrane that is not solubilized by Triton X-100 or Sarkosyl. A detergent solubilizes the material that is not covalently bound to it, e.g., lipidic components and un-cross-linked peptidoglycan away from the SPA bead. Since lipid II or un-cross-linked peptidoglycan is formed when the activities of either of the enzymes of PBPs is inhibited, the differences in the activities of the PBP inhibitors are seen only when the reaction products are captured differently by WGA-SPA beads in the presence and the absence of detergent.

Similarly, in the test to distinguish transpeptidase inhibitors from inhibitors of the other four enzymes, the difference lies in the two types of SPA beads (WGA beads versus type A PEI-WGA beads) used to capture the product. Both beads are made of PVT and are coated with WGA, but they are manufactured in different ways. In the type A PEI-WGA beads the WGA is bound to the bead via PEI, which must give it the altered properties that we have observed (Table 4). Type B PEI-WGA-SPA beads also have WGA linked to the bead via PEI, and these, too, can be used for selective identification of transpeptidase inhibitors (data not shown), but the buffer con-

ditions used for capture are different from those described here.

When the products of the reactions were captured by type A PEI-WGA-SPA beads, the β-lactams showed insignificant inhibition. This suggests that this bead type captures un-cross-linked peptidoglycan, possibly in addition to cross-linked peptidoglycan. In this respect the type A PEI-WGA-SPA bead is similar to the paper chromatogram, which does not distinguish nascent from cross-linked peptidoglycan. Inhibitors of the transpeptidase can be confirmed by analyzing the reaction products by paper chromatography. Since both cross-linked and un-cross-linked peptidoglycans remain at the origin of the chromatogram, transpeptidase inhibitors show no inhibitory activity when the quantity of radioactivity at the origin of the chromatogram is monitored, whereas transglycosylase inhibitors do. The other method used to confirm transpeptidase inhibition is analysis of the peptidoglycan formed by lysozyme digestion, followed by analysis of the digested products for the degree of cross-linking, but this is not amenable to the screening of large numbers of inhibitors (11). Also, inhibitors of the transglycosylase can be deduced by comparison of the results of the two tests that we have described.

Some inhibitors may act on more than one enzyme in the pathway; e.g., vancomycin is thought to act on the MraY and MurG, as well as the transglycosylase and the transpeptidase. With such compounds the action observed will be related to the inhibition of the initial enzymes in the pathway. For example, vancomycin would inhibit the MraY, and no radioactive product would be formed. Thus, vancomycin would appear to be an inhibitor of the MraY, MurG, or lipid pyrophosphorylase in the test used to select inhibitors of the PBPs (Fig. 4), This can be misleading, since in the intact cell vancomycin does not cross the membrane, and its primary target is thought to be the transpeptidase and/or transglycosylase (13); in a cell-free system it has access to and can inhibit the MraY and MurG as well. Similarly, transpeptidase inhibitors that also act on one of the upstream enzymes will not be detected as transpeptidase inhibitors in the other test. For this reason compounds that are found to be inhibitors of the transglycosylase or transpeptidase by either of these methods are likely to be specific to these enzymes, i.e., with no effect on the three enzymes earlier in the pathway. However, those that are classified as inhibitors of the MraY, MurG, and lipid pyrophosphorylase may also inhibit the activities of the PBPs.

The enzyme reaction, as described here, primarily measures the activity of PBP 1b. Other PBPs in the cell, e.g., PBP 1a, PBP 2, and PBP 3, are essential and would be good targets in the development of antibacterial agents; but inhibitors of these PBPs that do not bind to and inhibit the activity of PBP 1b will not be detected in this system. So far, with few exceptions, most inhibitors of the transpeptidases of other PBPs also inhibit PBP 1b, so there is a reasonable chance that novel inhibitors of the transpeptidase activities of other PBPs will also cross-react with PBP 1b.

In summary, we have described a method that can be used to distinguish inhibitors of the transglycosylase and/or the transpeptidase activity of *E. coli* PBP 1b from those of the other membrane enzymes involved in peptidoglycan synthesis. This is an enzyme assay for the transpeptidase and should detect inhibitors of a novel chemical class and with novel mechanisms of action. The assay can be performed in microtiter plates, requires little manipulation, and has the potential to discover novel antimicrobial agents.

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