Analysis of Periplasmic Enzymes in Intact Cultured Bacteria and Bacteroids of *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* biovar *phaseoli*

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Analysis of periplasmic enzymes of *Bradyrhizobium japonicum* or *Rhizobium leguminosarum* biovar *phaseoli* was hampered by the fact that only small amounts of marker enzyme activities were released from cells by osmotic shock or by lysozyme/EDTA treatment. However, up to 95% of total activity of certain periplasmic marker enzymes could be measured in intact cells by including 0.03% Triton X-100 in enzyme assays. Less than 5% of the cytoplasmic marker enzyme β-hydroxybutyrate dehydrogenase could be measured under these conditions, indicating that treatment with dilute detergent does not significantly perturb the cytoplasmic membrane. Various lines of evidence indicate that the dilute detergent treatment does not significantly alter wall structure but, instead, permits the assay of periplasmic enzymes by facilitating diffusion of substrates and products to and from the periplasmic space. For pyrophosphatase and phosphodiesterase, the proportion of total enzyme activity which could be measured in intact cells was higher in bacteroids than in bacteria at all Triton concentrations, suggesting that the outer membrane of bacteroids may be more permeable than the outer membrane of cultured bacteria. The effect of detergent on the hydrolysis of four disaccharides – sucrose, α,α-trehalose, maltose and lactose – was studied using intact cultured *R. leguminosarum* biovar *phaseoli*. The response of lactose hydrolysis to the addition of detergent was four-fold greater than the response of the other disaccharidase activities, indicating that a rhizobial lactase may be present in the periplasmic space.

**INTRODUCTION**

The identification and assay of periplasmic enzymes in *Rhizobium* bacteroids is of interest because they may play a role in the carbon and nitrogen nutrition of bacteroids. Although organic acids are thought to be the principal source of carbon for bacteroids, the microsymbiont may receive a variety of compounds from the host (Streeter & Salminen, 1985). To my knowledge, there are no published reports on the periplasmic enzymes in rhizobial bacteroids. The periplasmic enzymes alkaline phosphatase and cyclic phosphodiesterase can be released from *Escherichia coli* by osmotic shock or by treatment of cells with lysozyme and EDTA in the presence of a high concentration of sucrose (Malamy & Horecker, 1964; Neu & Heppel, 1965). The lysozyme/EDTA treatment was also found to release alkaline phosphatase from cultured *Rhizobium leguminosarum* bacteria (Glenn & Dilworth, 1979; de Maagd & Lugtenberg, 1986). However, in my preliminary studies, this technique was not successful in releasing cyclic phosphodiesterase from *R. leguminosarum* biovar *phaseoli* or *Bradyrhizobium japonicum* bacteroids; this is not surprising in view of the variation among genera, species and strains of bacteria in their response to lysozyme (Hughes et al., 1971; Osborn et al., 1972).

This paper describes the discovery that inclusion of very dilute Triton X-100 in assays with intact *B. japonicum* bacteroids makes it possible to measure nearly all of the phosphodiesterase activity. The utility of this detergent treatment was extended to the study of two other
periplasmic enzymes, alkaline phosphatase and pyrophosphatase, and intact bacteroids were compared to intact cultured bacteria using two different genera of rhizobia. Finally, the effect of dilute Triton on four different disaccharidases was determined using cultured *R. leguminosarum* biovar *phaseoli* grown with α,α-trehalose as the carbon source.

**METHODS**

*Bacterial and plant growth.* Cultures of *R. leguminosarum* biovar *phaseoli* (*'R. phaseoli'*) USDA 2667 and *B. japonicum* USDA 110 were obtained from the Nitrogen Fixation and Soybean Genetics Laboratory, Beltsville, Maryland, USA. Liquid cultures of *R. phaseoli* contained (mg l⁻¹): trehalose (2000), KNO₃ (800), CaCl₂, 2H₂O (6-6), MgSO₄·7H₂O (100), K₂HPO₄ (180), FeSO₄·7H₂O (0-13), biotin (0-12), thiamin.HCl (0-40), calcium pantothenate (0-50) and micronutrients as described by Manhart & Wong (1979). For the growth of *B. japonicum*, trehalose and KNO₃ were omitted and sodium glutamate (4-68 g l⁻¹) was used as a combined source of carbon and nitrogen (Tully, 1985). The pH of each medium was 7-0.

Seeds of *Phaseolus vulgaris* L. cv. Kentucky Wonder or *Glycine max* (L.) Merr. cv. Beeson 80 were planted in autoclaved silica sand and were inoculated at the time of planting with about 10⁵ cells of the appropriate bacterial strain per seed. Plants were grown in a greenhouse and were irrigated twice daily with nutrient solution lacking nitrogen. Nodules from plants 35 to 45 d of age were used for the isolation of bacteroids.

*Isolation of bacteria and bacteroids.* Bacterial cultures were used in late-exponential phase when OD₆₀₀ was generally between 0-6 and 0-9. This was usually after 3 d of growth for *R. phaseoli*, and after 7 d of growth for *B. japonicum*. Cultures (generally 500 ml) were centrifuged at 15000 g for 15 min, and the cells were resuspended in a wash medium. For *B. japonicum* the wash medium contained 5-4 g sucrose plus 370 mg CaCl₂, 2H₂O, 500 mg NaCl, 500 mg MgSO₄·7H₂O and 750 mg K₂SO₄ 1⁻¹. For *R. phaseoli* the wash medium contained 960 mg mannitol plus 1-0 g KCl and 120 mg MgSO₄·7H₂O 1⁻¹. The pH of each wash medium was 7-0. The components of the wash media were chosen to provide concentrations of nutrients and salts similar to those used in the growth media so that bacteria would not be subjected to osmotic shock during their isolation. Cells resuspended in a 20-fold or greater volume of wash medium were centrifuged at 12000 g for 10 min. The supernatant was discarded and washed cells were suspended in a small volume (generally 5 to 10 ml) wash medium. These suspensions were used directly for the assay of enzymes in intact bacteria. For the preparation of sonicated bacteria, Triton X-100 was added to give a final concentration of 0-05% (w/v) and cells were sonicated with a probe sonicator (Branson Cell Disruptor, model 350) with 100 W of power in a 50% pulse mode for 5 min at 0 °C.

For results reported in Figs 1 and 3, bacteroids were purified from soybean nodules using Percoll gradient centrifugation as described by Day *et al.* (1986). For all other experiments with bacteroids, nodules were chilled and ground in 0-15 M-Tris/HCl buffer pH 7-5 containing 0-15 M-mannitol, 1 mM-EDTA, 2 mM-DTE and 0-5% BSA. The crude homogenate was filtered through Miracloth and centrifuged at 44000 g for 10 min. The supernatant was discarded and bacteroids were suspended in the grinding buffer, diluted to about 25 ml per g fresh wt of nodule and centrifuged again at 44000 g for 10 min. Washed bacteroids were suspended in 0-15 M-Tris/maleate buffer pH 6-8 containing 0-15 M-mannitol and centrifuged again at 44000 g for 10 min. The twice-washed bacteroids were resuspended in a small volume (5 to 15 ml) of Tris/maleate/mannitol and assayed or sonicated as described above for cultured bacteria. The purity of bacteroids prepared by a similar procedure has been reported before (Salminen & Streeter, 1987).

*Enzyme assays.* All biochemicals were obtained from Sigma; purified Triton X-100 was obtained from Boehringer Mannheim. Protein concentration in bacteria and bacteroid preparations was determined by the bichinonic acid method (Smith *et al.*, 1985) using a commercial reagent (Pierce Chemical Co.).

Cyclic phosphodiesterase (also referred to as phosphodiesterase) was assayed in 68 mM-sodium acetate pH 6-0 containing 5 mM-MgCl₂, 1 mM-CoCl₂, and 0-25 mg bis(p-nitrophenyl)phosphate in a total volume of 1-0 ml (Neu & Heppel, 1965). Mixtures were incubated at 30 °C for 30 to 60 min and the reaction was stopped by the addition of 2-0 ml 0-1 M-NaOH. After mixing, 1-6 ml was centrifuged in a microfuge, and the A₄₁₀ of the supernatant was measured. The routine control lacked enzyme, but a zero-time control gave the same result. [The natural substrate for this enzyme is cyclic AMP, but the purified enzyme hydrolyses bis(p-nitrophenyl)phosphate, thus providing a more convenient assay (Neu, 1967). The use of the artificial substrate with crude protein preparations leaves the actual enzyme being assayed incompletely defined (Neu, 1967). In spite of this problem, numerous studies, including this one, show that this substrate provides a convenient assay for a periplasmic marker enzyme.]

β-Hydroxybutyrate dehydrogenase (EC1.1.1.30) was used as a marker for bacteroid cytoplasm (Day *et al.*, 1986; Planqué *et al.*, 1977; Reibach *et al.*, 1981). The enzyme was assayed in a mixture containing 85 mM-Tris pH 8-0, 1-2 mM-MgCl₂, 0-72 mM-NAD, and 12 mM-β-hydroxybutyrate in a total volume of 2-5 ml. ΔA₄₈₀ was determined using a Hewlett Packard 8452A spectrophotometer. Control ΔA (minus β-hydroxybutyrate) was essentially zero.

The assay of alkaline phosphatase (EC 3.1.3.1) was patterned after that used by de Maagd & Lugtenberg (1986), the main difference being the use of a lower Mg²⁺ concentration. The reaction mixtures contained 550 μl
pretreatment of purified bacteroids with 30% (w/v) sucrose and 1 mM-EDTA at room temperature for 10 min, centrifugation to collect the cells, and resuspension of the cells in ice-cold water. This procedure released only 2% of the phosphodiesterase from bacteroids. Also, sucrose interfered, but mannitol did not. The method finally adopted employed a reaction mixture containing 400 µl 0-15 m-Tris/maleate buffer pH 6-8 with 0-15 m-mannitol, 20 µl 0-1 m-MgCl₂ (Mg²⁺ is required for optimal activity: Rea & Poole, 1985), 20 µl 0-1 m-CoCl₂, 50 µl 100 mM-sodium pyrophosphate, and sample, detergent and water to give a total volume of 700 µl. The control was boiled bacteria or bacteroids. Mixtures were incubated at 30 °C for 30 min and the reaction was stopped by adding 100 µl of 0-5 m-H₂SO₄. Mixtures were transferred to microfuge tubes and centrifuged at 10000 g for 5 min. Six hundred microlitres of the supernatant was transferred to a clean test tube and mixed with 1-2 ml water, 0-75 ml 20% (w/v) sodium dodecyl sulphate, 0-3 ml 2-5% (w/v) ammonium molybdate in 2-5 m-H₂SO₄, and 0-15 ml 0-125% (w/v) l-aminoguanidine. The reaction was stopped by adding 100 µl of 1-4 g ‘Fiske & Subbarow Reducer’ (Sigma) per 10 ml water. See Dulley (1975) for details regarding the method of analysis. Mixtures were incubated at 37 °C for 30 min in a shaking water bath and A₆₆₀ was determined. Phosphate standards were made up in the pyrophosphatase assay mixture and were carried through the entire procedure.

The reaction mixture for estimating disaccharidase activities contained 700 µl 0-1 M-potassium phosphate buffer pH 6-6 (Salminen & Streeter, 1986) with 25 mM-disaccharide (sucrose, α,α-trehalose, maltose or lactose), and sample, detergent and water to give a total volume of 900 µl. The control was boiled bacteria. Mixtures were incubated for 30 or 60 min and the reaction was stopped by placing reaction mixtures in a boiling water bath for 5 min. The mixtures were then microfuged and 700 µl samples of the supernatant were transferred to clean test tubes for the analysis of glucose (Lloyd & Whelan, 1969).

Specific activities are reported on a protein basis even though the assay of protein in intact cells probably measured proteins other than surface and periplasmic proteins and would, therefore, not be strictly valid for calculating specific activity of periplasmic enzymes. For calculation of proportional activity, i.e. activity in intact cells as a percentage of total activity in sonicated cells, activity ml⁻¹ was used; a volume basis should be more accurate for this calculation because number of cells ml⁻¹ of sonicated and non-sonicated preparations was the same. For the conversion of specific activity to other units, the following factors were determined for cultured bacteria of R. phaseoli: 0-442 mg protein (mg dry wt)⁻¹; 0-141 mg dry wt (mg fresh wt of bacteria)⁻¹.

RESULTS

The osmotic shock procedure tried was similar to that of Neu & Heppel (1965) and involved pretreatment of purified bacteroids with 30% (w/v) sucrose and 1 mM-EDTA at room temperature for 10 min, centrifugation to collect the cells, and resuspension of the cells in ice-cold water. This procedure released only 2% of the phosphodiesterase from R. phaseoli bacteroids. For B. japonicum bacteroids, from 4 to 30% of total phosphodiesterase could be released by the osmotic shock treatment. However, various controls revealed that this release did not require pre-treatment with high sucrose concentration; i.e. a portion of the phosphodiesterase appeared to be very sensitive to release under a variety of resuspension conditions. Also, only 2 to 5% of a second periplasmic marker, pyrophosphatase, was released with the osmotic shock treatment. The lysozyme/EDTA procedure of Glenn & Dilworth (1979) released <9% of the phosphodiesterase from B. japonicum bacteroids and 12% of the phosphodiesterase from R. phaseoli bacteroids (means of two or more experiments). These procedures, which release high proportions of periplasmic enzymes from several bacteria (Hughes et al., 1971), did not appear to be useful with either B. japonicum or R. phaseoli bacteroids.

The assay of phosphodiesterase was attempted using intact bacteroids. Initially the effects of 75 mM-KCl, 0-02% Triton X-100 or 25 mM-EDTA on enzyme activity were determined, and both KCl and Triton treatments markedly increased the measured activity of phosphodiesterase. The effects of Triton were studied in more detail because the stimulation of activity was about 40% greater than for the KCl treatment and the maximum response was obtained at very low concentrations of detergent. About 90% of total phosphodiesterase activity was measured.
Fig. 1. Effect of Triton X-100 on phosphodiesterase (○) and β-hydroxybutyrate dehydrogenase (△) in purified, intact *B. japonicum* bacteroids. The mean and range of two observations from two independent bacteroid preparations is shown; for β-hydroxybutyrate dehydrogenase, range was within the plotted points. Maximum total activity was estimated using bacteroids sonicated in the presence of 0.05% Triton X-100 and assayed with 0.012 to 0.02% Triton X-100. Maximum specific activity [nmol min⁻¹ (mg protein)⁻¹] averaged 20.4 for phosphodiesterase and 7.17 for β-hydroxybutyrate dehydrogenase. However, for the proportional results shown, activity per ml of intact bacteroids was divided by activity per ml of sonicated bacteroids; a volume basis was used because of uncertainties involved in the analysis of protein in samples of intact cells (see Methods).

Fig. 2. Comparison of the effects of Triton X-100 on two periplasmic enzymes in *B. japonicum* bacteria and bacteroids. ○, Phosphodiesterase in bacteroids (○) and in cultured bacteria (●). □, Pyrophosphatase in bacteroids (□) and in cultured bacteria (■). Specific activity [nmol min⁻¹ (mg protein)⁻¹] for phosphodiesterase was 9.2 in sonicated bacteroids and 11.5 in sonicated bacteria; for pyrophosphatase, it was 94 in sonicated bacteroids and 98 in sonicated bacteria. Proportional activity shown in the figure was calculated as described in Fig. 1.

using intact bacteroids at only 0.015% Triton X-100 (Fig. 1). Activity of β-hydroxybutyrate dehydrogenase in the intact bacteroid preparation did increase in response to Triton, but activity relative to that in sonicated bacteroids was very low. These results were highly reproducible among bacteroid preparations, although different methods for bacteroid purification (e.g. Reibach *et al.*, 1981, vs Day *et al.*, 1986) gave slightly different patterns of response to Triton.

The overall pattern for the response of pyrophosphatase in *B. japonicum* bacteroids to Triton was similar to that for phosphodiesterase (Fig. 2). However, maximum activity was about 90% of total and a higher Triton concentration was required for the expression of maximum activity in intact bacteroids. In comparison to bacteroids, the proportional activity of both enzymes in cultured bacteria was lower at all Triton concentrations (Fig. 2, filled symbols). Specifically, only 10% of total activity was measured without detergent and a maximum of only 50–60% of total activity was measured at Triton concentrations ≥0.02%.

Only a small portion of phosphodiesterase was actually released from bacteroids in response to treatment with dilute Triton X-100 (Fig. 3). It is possible that most of the released enzyme came from damaged bacteroids, because enzyme activity released in the absence of Triton (9%) was almost as great as that released (12%) in the presence of Triton (Fig. 3). Thus, it appears that treatment of cells with dilute Triton does not significantly alter the structure of the outer membrane, but, instead, that Triton facilitates the exchange of substrates and products between
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Fig. 3. Release of phosphodiesterase from *B. japonicum* bacteroids treated with various concentrations of Triton X-100. Purified bacteroids were incubated at room temperature for 40 min and then collected by centrifugation at 6000 g. Bacteroids and supernatant samples were then assayed for phosphodiesterase with 0.02% Triton in the reaction mixture in order to provide maximum enzyme activity in bacteroids (see Fig. 1). Very similar results were obtained in a replicate experiment. •, Activity in bacteroids; ■, activity in supernatant; △, sum of the two activities. The specific activity in sonicated bacteroids was 15.3 nmol min⁻¹ (mg protein)⁻¹ in this experiment.

Fig. 4. Effects of Triton X-100 on two periplasmic enzymes in *R. phaseoli* bacteroids and cultured bacteria. ○, ●, Phosphodiesterase in bacteroids (○) and in cultured bacteria (●). □, ■, Pyrophosphatase in bacteroids (□) and in cultured bacteria (■). The mean and range for two different samples of cells is shown for phosphodiesterase. Specific activity [nmol min⁻¹ (mg protein)⁻¹] for phosphodiesterase averaged 10.5 in sonicated bacteroids and 11.4 in sonicated bacteria. For pyrophosphatase, specific activity was 90 in sonicated bacteroids and 238 in sonicated bacteria. Proportional activity shown in the figure was calculated as described in Fig. 1.

the periplasmic space and the reaction mixture. In other supporting experiments it was found that preincubation of intact bacteroids with dilute Triton did not give higher activity of phosphodiesterase or pyrophosphatase than was found when detergent was added at the time of substrate addition (data not shown). Also, identical Triton responses were obtained with either 30 min or 60 min incubations (data not shown). These results indicate that the effect of detergent is rapid; this would also be consistent with the idea that Triton facilitates diffusion of substrates and products but does not grossly alter cell wall structure.

Analysis of phosphodiesterase and pyrophosphatase in bacteroids from a different genus of symbiotic bacteria gave somewhat different results than those for *B. japonicum*. Specifically, about 55% of total phosphodiesterase could be measured in intact *R. phaseoli* bacteroids without the addition of detergent (Fig. 4). The response of phosphodiesterase to added Triton was somewhat erratic, but the pattern shown in Fig. 4 was reproducible. In contrast to the different pattern for phosphodiesterase, the response of pyrophosphatase activity to Triton in intact *R. phaseoli* bacteroids was similar to that found for *B. japonicum* bacteroids (open squares in Fig. 4 vs Fig. 2). Response curves for *R. phaseoli* cultured bacteria were muted relative to those for bacteroids; i.e. the response for phosphodiesterase was essentially flat (~50%) and the proportional activity for pyrophosphatase increased from 2% to only about 20% (Fig. 4). Thus, the comparison of bacteroids and cultured bacteria for pyrophosphatase was roughly similar to that shown in Fig. 2 for *B. japonicum*. 


Fig. 5. Effect of Triton X-100 on the activity of disaccharidase enzymes in intact *R. phaseoli* cultured bacteria. Results are expressed as a percentage of the total activity which could be measured when bacteria were sonicated prior to their assay. Maximum specific activities measured for the enzymes in sonicated bacteria [nmol substrate hydrolysed min\(^{-1}\) (mg protein)\(^{-1}\)] were: lactase (△), 10; invertase (☆), 68; maltase (○), 60; trehalase (▽), 34 (means for two experiments; range of the two values was about 5% of the mean).

The specific activity of alkaline phosphatase [generally <5 nmol min\(^{-1}\) (mg protein)\(^{-1}\)] was much lower than the specific activity of the other two periplasmic enzymes studied. This is probably due to the fact that this enzyme is induced only under low phosphate supply (Glenn & Dilworth, 1979), and the bacteria and bacteroids studied were apparently supplied with sufficient phosphate. A relatively high proportion (55 to 80%) of total alkaline phosphatase could be measured in intact cells in the absence of added detergent (data not shown), but because of the low activity present, the enzyme was not a useful periplasmic marker under the conditions studied here.

Because *R. phaseoli* used in this study was grown with trehalose as a carbon source and because trehalase has been reported as a periplasmic enzyme in a strain of *E. coli* (Boos et al., 1987), it was of interest to determine the response of disaccharidases to Triton detergent using intact *R. phaseoli*. Four disaccharides were tested: all disaccharide-hydrolysing activities were essentially nil with no added detergent, and all showed some activity in response to added detergent (Fig. 5). However, lactase, for which specific activity was relatively low, increased about fourfold more in response to Triton than did the other three enzymes. In a repeat experiment, centrifugation speeds used for collecting and washing bacteria were lowered and bacteria were resuspended as gently as possible in an attempt to avoid any damage to bacterial cell walls. Responses to Triton were still virtually identical to those shown in Fig. 5, indicating that the activities of invertase, maltase and trehalase were not due to damage to the cytoplasmic membrane. Because the response of all three enzymes to Triton was very similar (Fig. 5), it is suggested that there may be a general α-glucosidase in the periplasmic space, but most of the α-glucosidase activity did not appear to be in the same compartment as the lactase activity. It is unlikely that the difference among the response shown in Fig. 5 could be due to differences among the four substrates in diffusion into the periplasmic space because the outer membrane should be equally permeable to all four disaccharides (Benz & Bauer, 1988, Nikaido & Vaara, 1985).
The ineffectiveness of osmotic shock and the lysozyme/EDTA techniques for releasing periplasmic enzymes from bacteroids led to the discovery that periplasmic marker enzymes could be assayed in intact cells upon inclusion of detergent in the enzyme assay mixtures. The use of very dilute Triton X-100 was attractive because a high proportion of total enzyme/cell could be measured with only minor effects on the cytoplasmic marker, $\beta$-hydroxybutyrate dehydrogenase (Fig. 1). The lack of an effect of treatments on cytoplasmic enzymes is an extremely important point and is sometimes overlooked (Lall et al., 1989). Triton had almost no effect on assays of enzymes in sonicated cells (data not shown), so the detergent effect was not a direct effect of Triton on the catalytic activity of periplasmic enzymes.

In attempting to extend the 'Triton technique' to other conditions, it was found that the effect of dilute Triton varied depending on the enzyme assayed and on cell type. For example, there were clear differences between bacteroids and cultured bacteria, bacteroids having higher enzyme activity in the absence and in the presence of detergent (Fig. 2). The same result was obtained with *R. phaseoli*, but the clearest difference was for pyrophosphatase (Fig. 4). These differences between cultured bacteria and bacteroids are consistent with the suggestion that the bacteroid cell wall may be more permeable than the bacterial wall (van Brussel et al., 1977).

Another difference found was that between bacterial species. The clearest example was phosphodiesterase, which showed very different responses to Triton in *B. japonicum* and *R. phaseoli* (open circles in Figs. 2 and 4, respectively). Obviously, the accessibility of the enzyme to the substrate varies between the two species, but it is difficult to know if this is due to a difference in the location of the enzyme or to a difference between the outer membranes in their permeability to the bis(p-nitrophenyl)phosphate substrate. The fact that pyrophosphatase gave a 'typical' response to Triton in *R. phaseoli* suggests that the former explanation is correct. Furthermore, the fact that 45 to 60% of total phosphodiesterase could be measured in intact cells without the addition of detergent suggests that, at least in part, the enzyme is at or near the outer surface of the *R. phaseoli* cell. The fact that maximum activity of phosphodiesterase was achieved with 0.02% Triton whereas $>0.02\%$ Triton was sometimes required to give maximum pyrophosphatase activity may also indicate a different location of the two enzymes within the periplasmic space.

In spite of differences between enzymes, between species of rhizobia, and between bacteroids and cultured bacteria, the overall results support the general conclusion that it is possible to assay periplasmic enzymes using intact cells and that the use of dilute detergent greatly facilitates many of these assays. Triton was most consistently useful in the assay of pyrophosphatase and, in bacteroids, 70 to 90% of total enzyme activity could be assayed in intact cells using 0.03% Triton. Thus, in cases where the assay of periplasmic enzymes using intact cells is desired, pyrophosphatase may be the most generally useful marker. Because the effect of detergent is to increase diffusion of substrates and products, the technique may also have utility in the use of 'engineered' bacteria for the production of biochemicals, at least in cases where the last step in the synthesis is catalysed by a periplasmic enzyme.

The comparison of disaccharides was undertaken to illustrate that the 'Triton technique' can be used to probe the periplasmic space of intact bacteria for various enzyme activities. The results for $\alpha$-glucosidase activity are in agreement with recent evidence for the localization of maltase in the cytoplasm of a *Bacteroides* species (Anderson & Salyers, 1989). The unexpected result that lactase activity appears to have a periplasmic location is not in agreement with the literature which indicates that $\beta$-galactosidase is a cytoplasmic enzyme in *E. coli* (Malamy & Horecker, 1964; Neu & Heppel, 1965). However, virtually all of the work on $\beta$-galactosidase has been done with the artificial substrate nitrophenyl galactoside, and it is known that some $\beta$-galactosidases have activity with synthetic $\beta$-galactosides but will not hydrolyse lactose (Hartl & Hall, 1974). Even if the rhizobial enzyme is a 'nitrophenyl-$\beta$-galactosidase', different localization of the enzyme in these widely different bacteria is not unreasonable. However, it is not clear what might be the function of a periplasmic lactase in *R. phaseoli*. 

**DISCUSSION**

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