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Biodegradation of organophosphorus pesticides by surface-expressed organophosphorus hydrolase

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Organophosphorus hydrolase (OPH) was displayed and anchored onto the surface of *Escherichia coli* using an Lpp-OmpA fusion system. Production of the fusion proteins in membrane fractions was verified by immunoblotting with OmpA antisera. Inclusion of the organophosphorus hydrolase signal sequence was necessary for achieving enzymatic activity on the surface. More than 80% of the OPH activity was located on the cell surface as determined by protease accessibility experiments. Whole cells expressing OPH on the cell surface degraded parathion and paraoxon very effectively without any diffusional limitation, resulting in sevenfold higher rates of parathion degradation compared with whole cells with similar levels of intracellular OPH. Immobilization of these live biocatalysts onto solid supports could provide an attractive means for pesticide detoxification in place of immobilized enzymes, affording a reduced diffusional barrier.

Keywords: bioremediation, parathion, immobilized bioreactor

Synthetic organophosphorus compounds are used extensively as agricultural and domestic pesticides including insecticides, fungicides, and herbicides[1]. Due to environmental concerns associated with the accumulation of these pesticides in food products and water supplies, there is a great need to develop safe, convenient, and economically feasible methods for pesticide detoxification.

Current practices to detoxify organophosphate pesticides rely on chemical treatment, incineration, and landfills. Chemical methods, although feasible, are problematic in that large volumes of acids and alkali are produced and subsequently must be disposed. Landfills function adequately, but leaching of pesticides into surrounding soil and groundwater supplies is a cause for concern. Incineration, which is the most reliable method for destruction of these compounds, has met serious public opposition because of potentially toxic emissions and is economically restrictive.

Naturally occurring bacteria isolates capable of metabolizing organophosphorus compounds have received considerable attention as they provide the possibility of both environmentally friendly and in situ detoxification. Soil microorganisms such as *Pseudomonas diminuta* MG[2] and *Flavobacterium* sp.[3] possess high levels of a constitutively expressed enzyme, organophosphorus hydrolase (OPH), which has a broad substrate specificity (including the cleavage of P-O, P-F, and P-CN bonds) and catalyzes the hydrolysis of organophosphate pesticides, such as methyl and ethyl parathion, paraoxon, dursban, coumaphos, cyanophos, and diazinon as well as chemical warfare agents such as sarin and soman[4-7]. Enzymatic hydrolysis rates are 40–2450 times faster than chemical hydrolysis by 0.1 N NaOH at 40°C, and the activity of the enzyme is stable at temperatures up to 45–50°C (ref. 8).

Both native and recombinant OPH, immobilized onto nylon[9] (membrane, powder, and tubing), porous glass, and silica beads[8], have been applied as enzyme reactors for the detoxification of organophosphate pesticides. In most cases, the cost of this detoxification process depends heavily on the cost of purifying OPH. This problem can be eliminated if whole cells (either viable or nonviable), rather than enzymes, are immobilized onto the support (such as in an immobilized-cell bioreactor). The use of immobilized cells in a bioreactor does have disadvantages. The most serious potential problem is the mass-transport limitation of substrates and products across the cell membrane, as the outer membrane can act as a permeability barrier that may inhibit substrates from interacting with the enzymes contained within the cell. This diffusional resistance to mass transport can be reduced by treating cells with permeabilizing agents such as EDTA[10]. Not all enzymes are amenable to this treatment, and immobilized viable cells cannot be subjected to permeabilization[11]. This bottleneck could be eliminated if OPH was displayed on the surface of the cells.

A gene fusion system consisting of the signal sequence and the first nine amino acids of lipoprotein (Lpp) joined to a transmembrane domain from outer membrane protein A (OmpA) has been developed to anchor β-lactamase onto the cell surface[12]. We describe the construction and characterization of recombinant *Escherichia coli* strains that can anchor and display functional OPH onto the surface using this Lpp-OmpA gene fusion system. Cells expressing these Lpp-OmpA-OPH fusions were used as live biocatalysts to degrade parathion and paraoxon.

Results and discussion

Expression and localization of Lpp-OmpA-OPH. OPH is a membrane-associated protein whose N-terminus signal sequence is responsible for controlling cellular localization[13,14]. Recombinant OPHs produced in insect cells[15,16], *Streptomyces lividans*[17], and *E. coli*[18] are subject to different processing of this signal sequence. Soluble proteins targeted to the cell surface using the Lpp-OmpA fusion should be compatible with the protein-export machinery[12,19]; however, it is not clear whether inclusion of the OPH signal sequence (29 amino acids) would enhance translocation and proper processing of OPH across the cell membrane.

To investigate this, two recombinant plasmids were created: one encoding an Lpp-OmpA-OPH fusion enzyme containing the

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native OPH signal sequence (pOP131) and one without the signal sequence (pOP231). The resulting pOP131 and pOP231 plasmids had sizes of approximately 4.5 and 4.4 kb, respectively. Both plasmids contained an *lpp-lac* promoter ($P_{lpp\text{-}lac}$) enabling constitutive expression of the fusion enzymes.

The presence of plasmids pOP131 and pOP231 were lethal to *E. coli* JM105 cells at 37°C; however, 30°C proved to be a permissive temperature. Because protein folding is to a large extent temperature dependent[20,21], constitutive expression at 37°C may impair the protein folding mechanism, leading to incorrect insertion of the Lpp-OmpA-OPH fusion into the membrane. On the other hand, a lower temperature might retard the premature folding of the fusion protein, favoring proper translocation[21] and the high activities of OPH observed at 30°C.

Initial efforts were focused on characterizing the production of the Lpp-OmpA-OPH fusion proteins. Cultures were harvested in late exponential or stationary phase, lysed in a French pressure cell, and separated into soluble and cell envelope fractions by high-speed centrifugation[12]. The production of fusion proteins in the membrane fraction was shown by immunoblotting with OmpA antisera. Proteins of approximately 55 and 53 kD were detected from cells carrying constructs pOP131 and pOP231, respectively (Fig. 1A). No such fusion protein was detected in cells carrying plasmid pWM513 (ref. 22), which expresses OPH in the cytoplasm, or in the soluble fractions of cells carrying pOP131 or pOP231 (data not shown). The presence of additional (low intensity) lower molecular weight bands that cross-reacted with the OmpA antiserum is likely the result of limited proteolysis.

Even though immunoblot analysis (Fig. 1A) indicates that the quantity of Lpp-OmpA-OPH recombinant protein present in JM105(pOP131) and JM105(pOP231) cells is essentially the same, the activity of JM105(pOP231) was only about 5–10% that of JM105(pOP131) (Table 1), indicating the importance of the signal sequence in expressing active OPH on the surface. The majority of the OPH activity from JM105(pOP131) and JM105(pOP231) was found in the membrane fractions, while OPH activity from JM105(pWM513) was associated mostly with the soluble fraction, consistent with the data of Francisco et al.[12]

Extent of surface expression. Protease accessibility experiments were carried out to ascertain the presence of enzymatically active OPH on the cell surface. Cultures were incubated with and without proteinase K for one hour, and whole-cell activities were measured. Since proteinase K cannot readily diffuse across the cell membrane, degradation should only occur with proteins exposed on the surface. More than an 80% reduction in the OPH activity was observed in JM105(pOP131) cells incubated with proteinase K (Table 2). Thus, it appears that a substantial fraction of active OPH is fully exported across the membrane and anchored onto the cell surface. In contrast, no observable decline in OPH activity for proteinase K-treated cells was detected from JM105(pWM513). This result was confirmed by comparing the OPH activity specifically from the membrane fractions, which again indicated an 80% reduction in the proteinase K-treated JM105(pOP131) cells (Table 2). These observations are consistent with the expected surface localization of the fusion OPH.

Since only the OPH portion of the fusion protein is exposed on the cell surface, treatment with proteinase K should only degrade the OPH domain of the fusion protein without affecting the Lpp-OmpA moiety. A protein fragment of approximately 23 kD was detected from the protease-treated cells by immunoblotting (Fig. 1B). This polypeptide is likely the Lpp-OmpA protein fragment without the OPH moiety as it was not observed in proteinase K-treated cells carrying pWM513 (data not shown). Moreover, the fusion products of approximately 52–55 kD disappeared. Both of these results suggest that active OPHs are indeed expressed on the cell surface. The intensity of the OPH fusion protein bands were less than the proteinase K-degraded Lpp-OmpA fragment. We propose that binding of the OmpA antibody to the Lpp-OmpA portion was decreased due to the presence of OPH. Similar observations have been reported[23].

Whole-cell degradation of parathion and paraoxon. Because the outer membrane acts as a permeability barrier to restrict the passage of moderate to large substrates into the cell, diffusion of parathion and paraoxon may be restricted. This may represent a significant limitation to the large-scale detoxification of these pesticides by immobilized whole cells expressing intracellular OPH. To investigate this possibility, the rates of *p*-nitrophenol formation from parathion and paraoxon by intact cells expressing OPH from plasmids pMW513 and pOP131 were compared with their corresponding cell lysates. More than 90% of the OPH activity was

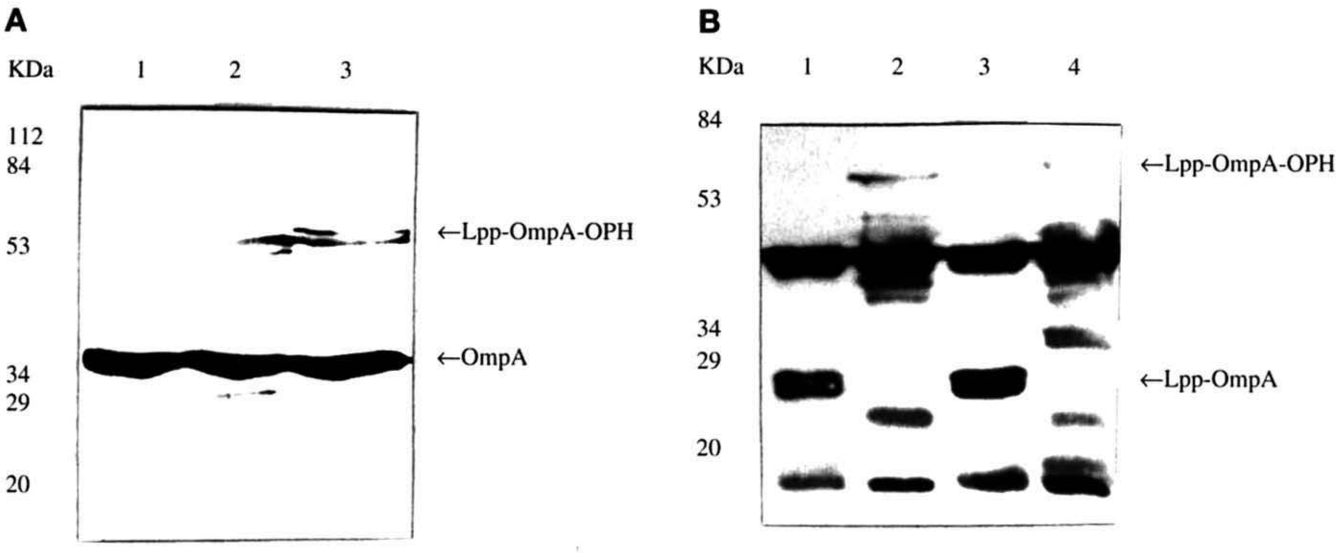

Figure 1. Western blot analyses of membrane fractions from JM105(pOP131), JM105(pOP231), and JM105(pWM513) cells. (A) Lane 1: pWM513; lane 2: pOP231; and lane 3: pOP131. Anti-OmpA antisera was used at 1:5000 concentration. The intense bands at 35 kD correspond to the native OmpA protein of *E. coli*. (B) Lane 1: pOP231 treated with proteinase K; lane 2: pOP231; lane 3: pOP131 treated with proteinase K; and Lane 4: pOP131.

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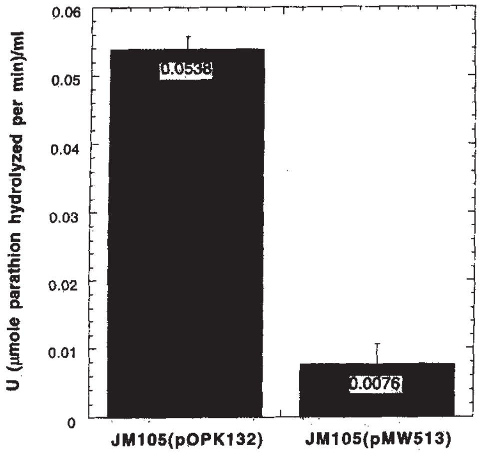

Figure 2. Whole cell activities with OPH expressed on the surface [JM105(pOPK132)] and intracellularly [JM105(pWM513)]. Whole cell activities were compared with equivalent levels of OPH activity (0.061 U/ml) from the cell lysates.

Table 1. OPH activity of *E. coli* strain JM105 carrying plasmids expressing OPH on the surface.

Plasmid	Salient features of plasmids	Activity*
pOP131	OPH signal peptide / *lpp-lac* promoter	0.078
pOP231	Similar to pOP131 but no signal peptide	0.010
pOPK132	Signal peptide / *tac* promoter / *lacI*^{q}	0.166

*micromoles parathion hydrolyzed per minute (U) per OD_{600} whole cells

Table 2. Surface expression of OPH as determined by protease accessibility and whole cell versus lysate assays.

	Percentage decrease in activity in proteinase K-treated cells		Whole cell activity (as % of lysate activity)	
Plasmid	Whole cell	Membrane Fraction	Parathion	Paraoxon
pOP131	82	77	93	90
pWM513	3	1	11	10

observed in whole cells expressing OPH from pOP131 compared with its lysate (Table 2). This contrasts markedly with only about 10% OPH activity in whole cells (compared with lysates) expressing OPH from pWM513. The low percentage of whole-cell activity in JM105(pWM513) probably arises from a substantial diffusional barrier for parathion and paraoxon across the cell membrane, because the addition of a permeabilizing agent, EDTA, increased whole-cell activity to a level similar to the lysates (data not shown). This problem, however, can be resolved by eliminating diffusion entirely with surface-expressed OPHs. From a practical standpoint, it is easy to envisage that the utility of live biocatalysts with active OPH expressed on the cell surface could replace more conventional cleanup strategies for organophosphorus pesticides. Immobilization of these live biocatalysts on a solid support may thus represent a very simple and attractive means of detoxifying pesticides in place of immobilized whole cells or immobilized enzymes by circumventing diffusional limitations, or (in the case of an immobilized-enzyme system) by reducing production costs and creating the potential for easy regeneration of the detoxification system.

Regulated expression of OPH on the cell surface. The JM105(pOP131) and JM105(pOP231) cultures were relatively unstable. Cells in liquid cultures of JM105(pOP131) and JM105(pOP231) tended to aggregate, and there was visible evidence of cell lysis. Petri plate cultures of these cells had to be restreaked weekly to keep the cultures viable. Long-term storage on plate or in frozen stock resulted in a significant reduction in OPH activity. It has been reported that the expression of a plasmid-specified exported protein can cause plasmid instability[24]; a similar phenomenon may have occurred here. Restriction enzyme digests of plasmids isolated from liquid cultures derived from one-month-old petri dish cultures of pOP131 resulted in a variety of fragments with sizes differing markedly from those observed initially (data not shown).

This instability problem was resolved, however, when a tightly regulated, inducible *tac* promoter was used to express the OPH fusions on the cell surface (pOPK132). Cells carrying this plasmid did not show detectable OPH activity prior to induction. As a result, the extensive cell aggregation and cellular instability observed in the JM105(pOP131) cultures were not observed in the JM105(pOPK132) cultures. The JM105(pOPK132) cultures were also viable and active when grown at 37°C. OPH production was quickly induced within one hour upon the addition of IPTG. Whole cell activity for two-day-old cultures of JM105(pOPK132) was almost twice that observed from JM105(pOP131) (Table 1). Again, almost 85% of the OPH activity was accessible from the whole cells of JM105(pOPK132) as compared with the cell lysates. More importantly, with similar levels of OPH expressed on the surface or intracellularly (normalized by the OPH activity in the cell lysates), whole cells of JM105(pOPK132) had sevenfold higher activity than JM105(pWM513) (Fig. 2), illustrating that surface-expressed OPH affords greater degradation than intracellularly-expressed OPH. Therefore, an improved whole cell technology for organophosphorus pesticide biodegradation has been realized.

Active OPHs anchored onto the cell surface are less likely to be subject to degradation from intracellular proteases, a condition that is commonly encountered during starvation. It is conceivable that resting cultures with surface-expressed OPH may enable pesticide detoxification to be sustained much longer than resting cultures with intracellularly expressed OPH. This, in conjunction with the improved degradation by whole cells of JM105(pOPK132), suggests that recombinant cells with surface-expressed OPH could be effective for large-scale and long-term detoxification of organophosphorus pesticides. We are currently investigating the degradation of a wide variety of pesticides in a bioreactor with these recombinant cells immobilized onto a solid support.

Experimental protocol

Strains, plasmids, and culture conditions. Except as otherwise noted, routine molecular biology procedures were used[25]. *E. coli* strains JM105 (F′ *traD36 lacI*^{q} *Δ(lacZ)M15 proA*$^+$*B*$^+$|*thi rpsL* (Str^r) *endA sbcB15 sbcC hsdR4*($r_k^- m_k^+$) *Δ(lac-proAB)*) and DH5α F′ (F′ *ϕ80dlacΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17*($r_k^- m_k^+$) *supE44*λ^- *thi-1 gyr*A96 *relA1*) were used in this study. Routine transformations were performed with *E. coli* DH5α F′. *Flavobacterium* sp. strain ATCC 27551 was obtained from the American Type Culture Collection. For isolation of plasmid DNA from *Flavobacterium*, the protease cell lysis and plasmid isolation procedure of Currier and Nester[26] was used except that the DNA shearing step and the purification on cesium chloride-ethidium bromide gradients were omitted. The *lpp-ompA* fusion was PCR amplified from plasmid pTX210 (ref. 19) using primers and conditions as described by Francisco et al.[19] The resulting PCR fragments were subcloned into pUC18 (Pharmacia, Piscataway, NJ). Plasmids pWM513 and pJK33 (ref. 22), which contain the *opd* gene from *Flavobacterium* sp. were used as a control for the production of native OPH in the cytoplasm. *E. coli* cells were cultivated in LB medium (10 g/L Difco tryptone, 5 g/L Difco yeast extract, 10 g/L NaCl, 3 g/L K_2HPO_4, and 1 g/L KH_2PO_4, pH 7.0). Ampicillin was added to 50 mg/L for selection.

Construction of Lpp-OmpA-OPH fusions. PCR was used to amplify a DNA fragment containing (1) the *lpp-lac* promoter and (2) the signal sequence and first nine amino acids of Lpp joined to a transmembrane domain (amino acids 46–159) from OmpA using plasmid pTX210 as the

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template[19]. Two oligonucleotides, Lpp-OmpA1 (dGGGGCATGCCTGGATAACCAGAAGCAATAAAAAA), and Lpp-OmpA3 (dGGCCGGGTACCGAAATTCCGTTGTCCGGACGAGTGCCGAT) served as primers for the 5′ and 3′ end of the fragment, respectively. The resulting 700-bp fragment ($\text{P}_{\text{lpp-lac}}$-*lpp-ompA*) was cut with KpnI and SphI, and ligated into similarly digested pUC18 to give pOmpA1. JM105 was used as the recipient cell line for transformations owing to its *lacI^q* genotype. The *opd* gene coding for OPH was PCR amplified using the plasmid preparation of *Flavobacterium* sp. strain ATCC 27551. Three oligonucleotide primers were used: opd1) dGGGGGTACCCGGGATGCAAACGAGAAGGGTTGTGCTCA; opd2) dGGGGGTACCCGGGTCGATCGGCACAGGCGATCGGATCAAT; and opd3) dGG-GGAATTCAAGCTTCCAAAAAAAAGCCCGCTCATTAGGCGGGCTGCGTCATGACGCCCGCAAGGTCGGTGACAAG. To generate an in-frame fusion between *opd* and *lpp-ompA*, both the larger *opd* (~1100 bp with the signal sequence of OPH amplified with primers opd1 and opd2) and the smaller *opd* (~1000 bp without the signal sequence of OPH amplified with primers opd2 and opd3) PCR fragments were digested with Kpn1 and EcoR1, and ligated into similarly digested pOmpA1 to give pOP131 and pOP231, respectively. The resulting vectors contain the pUC replication origin, an ampicillin marker, and the *lpp-ompA-opd* fusion preceded by the *lpp-lac* promoter. Expression of the respective Lpp-OmpA-OPH proteins is controlled by the *lpp-lac* promoter. To construct an inducible construct for the Lpp-OmpA-OPH fusion, PCR was used to amplify Lpp-OmpA fragment without the *lpp-lac* promoter using plasmid pTX210 as the template. Two oligonucleotides, Lpp-OmpA2 (dGGGGAATTCCTCTAGAGGGTATTA-ATAATGAAAGCT), and Lpp-OmpA3 served as primers for the 5′ and 3′ end of the fragment, respectively. The resulting 500-bp fragment was cut with EcoRI and KpnI, and ligated into similarly digested pUC18 to give pOmpA2. The ~1100-bpPCR fragment of *opd* was digested with Kpn1 and HindIII and ligated into similarly digested pOmpA2 to give pOP132. Finally, the plasmid pOPK132 was constructed by inserting an *Eco*R I/*Hind* III fragment from pOP132 into similarly digested pKQV4 (ref. 27), a *lacI^q* derivative of pKK223-3 (Pharmacia). The resulting vector contains the pBR replication origin, an ampicillin marker, the *lacI^q* gene, and the *lpp-ompA-opd* fusion preceded by the *tac* promoter. Expression of Lpp-OmpA-OPH from pOPK132 is tightly regulated by the *tac* promoter due to the presence of the *lacI^q* gene on the plasmid.

Organophosphorus hydrolase assay. Cells from 2-day-old cultures or membrane fractions were collected by centrifugation and resuspended in 15% (w/v) sucrose; 15 mM Tris-HCl (pH 7.8). Assays were conducted in 1.5-ml disposable methacrylate cuvettes (Fisher, Tustin, CA). For each assay, 900 µl of test sample (~ 1 $\text{OD}_{600\text{nm}}$ cells or lysate from ~ 1 $\text{OD}_{600\text{nm}}$ cells) were combined with 100 µl of 6.5 mM parathion (Supleco, Bellefonte, PA) or 10 mM paraoxon (Sigma) in 10% methanol. Reaction mixtures were incubated at room temperature or 30°C (as indicated) during which time the changes in absorbance (410 nm) were monitored. Activities are expressed as micromoles of parathion or paraoxon hydrolyzed per minute, per $\text{OD}_{600\text{nm}}$ whole cells (e_{410}=16,500 M^{-1} cm^{-1} for *p*-nitrophenol). Cell lysates were prepared by adding 5 µl of 10 mg/ml lysozyme solution to the test sample (in 15% sucrose; 15 mM Tris, pH 7.8). Following a 10-min incubation at room temperature, the samples were sonicated for 10 sec, then assayed for OPH activity as described above.

Cell fractionation. The procedure of Francisco et al.[12] was used for the fractionation of inner and outer cell membranes. One milliliter fractions were taken from tops of the sucrose gradients, and assayed directly for OPH activity. Samples to be analyzed by SDS-PAGE and/or Western blot analysis were diluted and centrifuged as described[12].

Proteinase protection/sensitivity. To demonstrate the localization of the Lpp-OmpA and OPH moieties, a proteinase-K treatment was used. Samples of JM105(pOP131), or JM105(pWM513) (~ 1 $\text{OD}_{600\text{nm}}$ centrifuged and resuspended in 900 µl of 15% [w/v] sucrose; 15 mM Tris-HCl, pH 7.8) were incubated with 5 µl of 20 mg/ml proteinase-K for 60 min at 30°C. Following the incubation, 10 µM phenylmethylsulfonylfluoride was added. Proteinase-treated cells were compared with untreated cells for their abilities to degrade parathion (as described above).

Western blot analysis. Samples (10 µl) of 4× concentrated cells were combined with 5 µl of disruption buffer[28] and boiled for 5 min. Samples were then electrophoresed through 12.5% (w/v) acrylamide SDS-PAGE gels prior to staining[28] or Western blot analysis. Western blot analysis was performed using a BioRad Immun-Blot GAR-AP kit (BioRad, Hercules, CA). Antibody prepared against OmpA was generously donated by G. Georgiou. Prestained low-range protein markers (BioRad) were used for estimation of protein molecular weights.

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