isolated on oligo(dT) cellulose columns (*19*). Oligonucleotides complementary to PEPCK and calmodulin were end-labeled with [$\gamma$-$^{32}$P]ATP, whereas an oligonucleotide complementary to CAT was internally labeled with [$\alpha$-$^{32}$P]dATP (deoxyadenosine triphosphate) and [$\alpha$-$^{32}$P]dCTP (deoxycytidine triphosphate). Labeled primers were added to 10 $\mu$g of poly(A)$^+$ RNA and extended with reverse transcriptase (*20*). Reaction products were separated on a denaturing polyacylamide-urea gel and analyzed by autoradiography.


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21. Each PEPCK-TKCAT construct (30 $\mu$g) was transfected in duplicate into H4IIE cells at 40 to 70% confluency in 75-cm$^2$ culture dishes as described (*15*). Six hours after transfection the cells were treated with medium containing 20% (v/v) dimethyl sulfoxide for 5 min, washed, and then incubated for 18 hours in the presence or absence of insulin (10 nM). Cells were harvested and CAT activity was assayed as in Fig. 1.
22. DNA fragments, labeled with [$\alpha$-$^{32}$P]dATP and the Klenow fragment of *Escherichia coli* DNA polymerase I, were incubated with 10 $\mu$g of rat liver nuclear extract, at room temperature for 15 min in the presence of 10 mM Hepes at pH 7.8, 1 mM spermidine, 10 mM dithiothreitol, 50 mM NaCl, 2 $\mu$g of poly(dI/dC), 10% glycerol (v/v), and 0.1% NP-40 (v/v). Unlabeled competitor DNA fragments (a 100-fold molar excess) were added for competition analyses. Samples were loaded onto a 6% polyacrylamide gel and separated by electrophoresis (150 V) at 4°C for 2 hours in a buffer containing 25 mM tris, 190 mM glycine, and 1 mM EDTA (*23*). Gels were dried, and binding was analyzed by autoradiography.


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# Protection Against Mucoid *Pseudomonas aeruginosa* in Rodent Models of Endobronchial Infections


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**Chronic endobronchial infection with mucoid *Pseudomonas aeruginosa* accounts for much of the morbidity and mortality in patients with cystic fibrosis (CF). Reduced morbidity is observed when infection is absent. Clinical investigations have implicated opsonizing antibody specific for the mucoid exopolysaccharide (MEP) surrounding these bacteria as a potential immunologic protective mechanism, whereas nonopsonizing antibody to MEP is not protective. Mice and rats immunized with doses of MEP that elicited opsonizing antibody had reduced levels of infection compared with nonimmune controls after intratracheal challenge with mucoid *P. aeruginosa* enmeshed in agar beads. Doses of MEP that elicited nonopsonizing antibody were not protective. Parallel experiments in which passive transfer of polyclonal and monoclonal opsonizing and nonopsonizing antibody were used yielded similar results. These data indicate that MEP-specific opsonizing antibody can protect against chronic *P. aeruginosa* infection in this model of disease.**


THE ABILITY OF MICROORGANISMS to establish chronic infections in immunocompetent individuals is an enigma of host-parasite interactions. Such microorganisms must somehow subvert or escape the immune defenses for long periods. In some instances, such as the acquired immunodeficiency syndrome (AIDS), the human immunodeficiency virus directly attacks the cells of the immune system (*1*). Alternatively, organisms may hide inside cells and thus avoid host defenses (*2*) or may change their antigenic structures and produce new antigens that do not react with antibody produced against previously expressed antigens (*3*). However, none of these mechanisms has been identified as critical to the maintenance of chronic lung infections in patients with CF. These infections, mostly caused by mucoid strains of *P. aeruginosa*, cause the vast majority of the morbidity and ultimate mortality early in life (*4*). Eighty-seven percent of CF patients develop respiratory tract colonization with *P. aeruginosa* by their 15th birthday (*5*). However, before the appearance of *P. aeruginosa* in sputum cultures of young patients, and among those older CF patients without *P. aeruginosa* colonization, there is greatly reduced morbidity (*6*). Most CF patients respond immunologically to their *P. aeruginosa* infection by producing antibodies against a large number of bacterial antigens (*7*). Among these antibodies are those directed against the outer polysaccharide coat, known both as alginate and as mucoid exopolysaccharide (MEP) (*8*). Chronically colonized CF patients produce antibodies to MEP that are not opsonic, that is, they are incapable of mediating phagocytosis and of killing bacteria in the presence of human leukocytes and complement (*9*). In contrast, 14 of 16 older (>12 years) CF patients who had managed to escape chronic *P. aeruginosa* infection had MEP-specific antibodies that were capable of mediating bacterial killing via opsonization (*9*).

Although no animal model of CF yet exists, chronic lung infections can be established in laboratory animals by administering bacteria enmeshed in agar beads (*10*). When purified MEP [prepared as described in (*11*)] was used to immunize mice and rats, opsonizing antibody was elicited after two doses of 1 to 10 $\mu$g per animal. This activity can be quantitated as an opsonic-killing index, which represents the percentage of bacteria killed in the presence of the opsonizing antibody compared to normal serum. Indices of >45% usually represented statistically significant ($P > 0.05$) killing and are considered positive. In contrast, doses of ≥40 $\mu$g per animal elicited only nonopsonizing antibody (<45% killing), and these animals did not produce opsonizing antibody when boosted from 1 to 8 weeks later with 1-$\mu$g doses (*11*). An exception was when only the highest molecular-sized [estimate of the distribution coefficient ($K_{av}$) = 0.05 on a Sepharose CL 4B column] polymers of MEP were used as immunogens. These polymers elicited opsonizing antibody in mice at doses of 50 $\mu$g (*11*). Thus, we were able to selectively induce either nonopsonizing or opsonizing antibody in animals and to study their effect on protective immunity.

We initially immunized adult, female rats with two doses 7 days apart of either 10 or 100 $\mu$g of MEP, which induced opsonizing and nonopsonizing antibodies, respectively. Five days after the second dose, two challenge strains of mucoid *P. aeruginosa* enmeshed in agar beads by the method of Cash *et al.* (*10*) were used to infect the rats: strain 2192, from which the MEP was isolated, and strain 258, which consistently produces chronic infections in these animals (*12*). After 21 days the animals were killed and their lungs were removed for quantitative and qualitative bacterial enumeration (*13*). Compared with saline controls, the rats immunized with doses of MEP that elicited high (≥91%) mean opsonic-killing indices in their pooled serum had significantly ($P < 0.02$) fewer lungs that yielded growth of


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mucoid *P. aeruginosa* (Table 1). In contrast, animals immunized with 100 μg of MEP, which elicited low (≤9%) mean opsonic-killing indices in the serum pool, had lungs yielding growth of mucoid *P. aeruginosa* at a level comparable to nonimmune controls (*14*). Immunoglobulin G (IgG) and IgM antibody titers, which measure the levels of total (binding) antibody to MEP, were not different between low- and high-dose immune groups as measured by enzyme-linked immunosorbent assay (ELISA) (*8*).

Similar experiments were then done in mice (*15*). Animals were killed after 7 days, since preliminary experiments indicated that clearance of bacteria from the lungs was complete by 4 days after immunization and that the mean number of organisms per lung did not vary by more than 15% over a period of 5 to 35 days, when samples were taken every 3 to 4 days. Confirming the findings in rats, mice immunized with low doses (1 μg) of MEP, which elicited a mean opsonic-killing index of 96% in the serum pooled from these animals, had significantly ($P = 0.0079$) fewer lungs that yielded mucoid *P. aeruginosa* when cultured, compared with the saline controls. Animals given a 50-μg dose of MEP, which elicited a mean opsonic-killing index of only 11% in the pooled serum, had comparable numbers of mucoid *P. aeruginosa* in the cultures of their lung homogenates as did saline-immunized controls. We also tested the protective efficacy of a lot of MEP, designated lot 2, which is made up solely of the highest molecular-sized ($K_{av} = 0.05$) polymers of MEP. This material induced an opsonic-killing index of 82% after two 50-μg doses (*11*) and afforded protection against chronic lung infection (Table 1).

**Table 1.** Protection of rats (after 21 days) and mice (after 7 days) against chronic mucoid *P. aeruginosa* infection after immunization with MEP. After immunization, the opsonic-killing index was determined as the percentage of surviving bacteria killed by a 1:8 serum dilution (*18*). The *P* values were calculated by cross-tabulation (χ-squared with the continuity correction) by comparing saline immunized controls with the indicated group. NS, not significant, $P > 0.025$, with the Bonferroni adjustment (*22*) of the significance level of $\alpha = 0.05$ for experiments with two experimental groups per control group, and $P > 0.017$ for experiments with three experimental groups per control group.

| Immunogen | Dose (μg) | Opsonic-killing index | Challenge strain | No. infected/total | *P* value |
|---|---|---|---|---|---|
| | | *Rats* | | | |
| Saline | | 0 | 2192 | 8/9 | |
| MEP | 10 | 98 | | 2/8 | 0.0152 |
| MEP | 100 | 8 | | 7/9 | NS |
| Saline | | 0 | 258 | 13/15 | |
| MEP | 10 | 91 | | 3/18 | 0.0003 |
| MEP | 100 | 9 | | 15/18 | NS |
| | | *Mice* | | | |
| Saline | | 0 | 258 | 14/18 | |
| MEP lot 1 | 1 | 96 | | 3/13 | 0.0079 |
| MEP lot 1 | 50 | 11 | | 13/14 | NS |
| MEP lot 2 | 50 | 82 | | 2/15 | 0.0031 |

**Table 2.** Lack of protection against chronic mucoid *P. aeruginosa* strain 258 infection in mice after immunization with heat-killed cells of either mucoid or nonmucoid revertant strain 2192 compared with protection mediated by immunization with purified MEP. The opsonic-killing index was determined as the percentage of surviving bacteria killed by a 1:8 serum dilution (*18*), and the antigenic specificity of the opsonins was determined by adsorption and inhibition experiments as described (*11*). The *P* values were calculated by cross-tabulation (chi-squared with the continuity correction) comparing saline controls with the indicated group. HK MPA, heat-killed mucoid *P. aeruginosa*; HK NMPA, heat-killed, non-MEP producing *P. aeruginosa*; NS, not significant; $P > 0.017$ with the Bonferroni adjustment (*22*) of the significance level of $\alpha = 0.05$.

| Immunogen | Dose | Opsonic-killing index | Specificity of opsonins | No. infected/total | *P* value |
|---|---|---|---|---|---|
| Saline | | 0 | | 10/10 | |
| MEP lot 1 | 1 μg | 92 | MEP | 2/10 | 0.0014 |
| HK MPA | $10^9$ | 88 | Not MEP | 10/10 | NS |
| HK NMPA | $10^9$ | 79 | Not MEP | 9/10 | NS |

Although CF patients chronically colonized with MEP-producing strains of *P. aeruginosa* do not respond to infection with MEP-specific opsonizing antibody, they do respond with antibodies that can mediate opsonic killing of mucoid *P. aeruginosa* in vitro (*9*). Presumably these antibodies are specific for antigens other than MEP, since they can be adsorbed from patient's sera with non-MEP–producing strains of *P. aeruginosa* (*9*). This adsorption process does not reduce the titer of the nonopsonizing, MEP-specific antibodies in these sera (*9*). Opsonizing antibodies directed at antigens other than MEP can be elicited by immunizing mice with high doses ($10^9$) of heat-killed mucoid and nonmucoid *P. aeruginosa* cells. At this dose of heat-killed cells, mucoid *P. aeruginosa* elicit only nonopsonizing antibody to MEP (*11*). When heat-killed mucoid and nonmucoid *P. aeruginosa* cells were given to mice in two doses 7 days apart and the mice were challenged 5 days later with mucoid cells enmeshed in agar beads, no protection was seen (Table 2). The sera from these animals did contain non-MEP–specific antibody that had in vitro opsonic-killing indices comparable to those in sera obtained from animals immunized with 1 μg of MEP. In the same experiment, immunization with 1 μg of purified MEP protected 80% of the animals. Thus, in spite of having in vitro opsonic activity elicited by immunization with heat-killed bacteria, the lack of specificity of these opsonins for MEP made them incapable of clearing bacteria from the lung. Analysis of all of the data from rats and mice (excluding the saline controls) indicated that 12 of 64 (19%) immunized animals with MEP-specific mean opsonic-killing indices of ≥82% had lung cultures that yielded growth of mucoid *P. aeruginosa* when killed, compared with 54 of 61 (89%) immunized animals that had MEP-specific mean opsonic-killing indices of ≤11% ($\chi^2 = 58.31$, $P = 0.0001$).

To confirm that the opsonizing antibody elicited by immunization was critical for the lung clearance observed, we performed passive transfer experiments with normal human sera containing high titers of nonopsonizing antibody, sera from humans immunized with MEP who had responded by producing opsonizing antibody, and two murine IgG2b/κ monoclonal antibodies (*16*) that differed in their opsonizing activities (Table 3). Highly significant protection was afforded by administering sera or monoclonal antibodies (MAbs) with opsonizing activity.

Examination of the lungs of infected animals showed that 82% of animals given doses of MEP eliciting nonopsonizing antibody had a mottled red and pink appearance with areas of hemorrhage. Histologic examination of a subset of these animals indicated that infected animals had mild or moderate multifocal, mononuclear peribronchial infiltrates usually with focal, purulent bronchiolitis. In some animals, bronchopneumonia was observed. Neutrophil-coated agar beads

could be observed in the airways (Fig. 1, A to C) surrounded by areas of inflammation. In contrast, animals immunized with MEP, so as to elicit opsonizing antibody, usually had only slightly mottled-appearing lungs, with 23% showing areas of hemorrhage. Upon histologic analysis of a subset of these animals, most of the specimens showed no more than a diffuse, mild mononuclear perivascular infiltrate. The tissue surrounding the neutrophil-coated agar beads in the airways appeared healthy (Fig. 1, D to E).

These data support the potential prophylactic efficacy for MEP-specific opsonizing antibody to protect CF patients against chronic infections caused by mucoid *P. aeruginosa*. We currently have no information as to why antibodies with comparable in vitro opsonic properties have differing in vivo protective properties. Data indicate that the different in vitro activities of MEP-specific opsonizing and nonopsonizing antibodies is because of disparate abilities to deposit fragments of the third component of complement (C3) onto the bacterial surface (*17*). Since opsonic killing of mucoid *P. aeruginosa* is completely dependent on the activation of complement (*18*), this correlation explains the differing activities of these MEP-specific antibodies.

Previous studies by Woods and Bryan (*19*) have suggested that protection after immunization with MEP was strain-dependent, and in some animals an undesired side effect could be development of immune complexes as a consequence of MEP immunization. However, they did not determine the opsonic activity of the antibodies elicited by the two MEP preparations they used. Given that they immunized rats with doses of 100 μg of MEP, which would have elicited nonopsonizing antibody, it appears that their inability to protect most of the animals was because of elicitation of these nonopsonizing antibodies. Although we did not look for immune complexes in the animals immunized with high doses of MEP, it is possible that in the absence of MEP-specific, opsonizing antibody, the nonopsonizing antibodies could contribute to the pathology of chronic *P. aeruginosa* infection by forming immune complexes, as suggested by Woods and Bryan. In addition, Gilleland *et al.* (*20*) have demonstrated that immunization with *P. aeruginosa* outer membrane protein F protects rats from chronic *P. aeruginosa* infection, using as challenge strains nonmucoid cells expressing smooth lipopolysaccharide O side chains.

The major barrier that prevents testing of MEP as a vaccine in CF patients is its poor immunogenicity in humans (*21*). This is likely because the adults immunized so far have preexisting nonopsonizing antibody, which may either directly interfere with the induction of opsonizing antibody or be a marker for an inability to produce opsonizing antibody because of some immunologic suppressor mechanism. About 20% of adults immunized with 100 μg of MEP have made long-term opsonizing antibody responses. Mice immunized with high doses of MEP, which elicit nonopsonizing antibody, are also unable to respond to lower doses of MEP with opsonizing antibody, and this inability to respond can be transferred to naïve mice by T cells obtained from the spleens of animals given high doses of MEP (*11*). However, certain preparations of MEP, comprised solely of the highest molecular weight polymers produced by the bacteria in broth cultures, appear to have enhanced immunogenicity in terms of eliciting opsonizing antibody at high doses as well as eliciting opsonizing antibody in animals with preexisting nonopsonizing antibody (*11*). Alternate means of eliciting opsonizing antibody, such as protein-polysaccharide conjugates, may be able to consistently induce these antibodies in humans. Once a reliable means of inducing opsonizing antibody to MEP is found, vaccine trials of this preparation in patients with CF could be undertaken.

**Table 3.** Protection against chronic lung infection (7 days) in mice caused by mucoid *P. aeruginosa* strain 258 after passive transfer (intraperitoneally) of 0.5 ml of sera or 500 μg of MEP-specific MAbs with and without opsonizing activity 2 hours before challenge. The opsonic-killing index was determined as the percentage of surviving bacteria killed by a 1:8 serum dilution or 500 μg of MAb (*18*). The *P* values were calculated by cross-tabulation of the experimental with the indicated control group (chi-squared with continuity correction for studies with polyclonal sera, and Fisher's exact test for studies with MAbs). NHS, normal human serum; IHS, immune human serum; NS, not significant; $P > 0.025$ with the Bonferroni adjustment (*22*) of the significance level of $\alpha = 0.05$.

| Preparation transferred | Opsonic-killing index | No. infected/ total | *P* value |
|---|---|---|---|
| Saline | 0 | 16/21 | |
| NHS | 0 | 19/21 | NS versus saline |
| IHS | 98 | 6/20 | 0.008 versus saline<br>0.0003 versus NHS |
| MAb-16 | 3 | 6/7 | |
| MAb-8)5/31 | 84 | 1/7 | 0.02 versus MAb-16 |

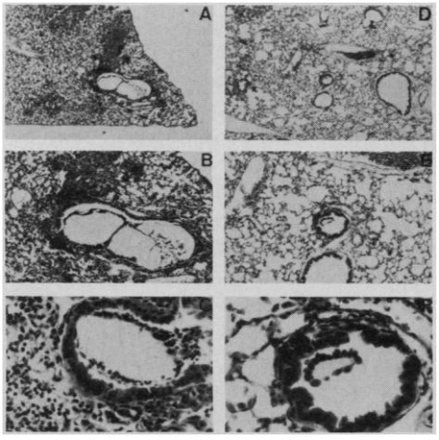


**Fig. 1.** Hematoxylin- and eosin-stained lung sections obtained from mice immunized with doses of MEP, which elicit (**A** to **C**) nonopsonizing antibody or (**D** to **F**) opsonizing antibody. In each section an area of lung containing an airway with an agar bead is shown. In (A) to (C) a cellular infiltrate is present in tissue surrounding the airways in which the agar beads containing organisms have lodged. In these sections, significant pulmonary parenchymal consolidation is present. In sections taken from an immune animal (D to F), cellular infiltrates within alveoli and surrounding airways containing beads are markedly less intense. (A) and (D), ×40; (B) and (E), ×100; (C) and (F), ×400.

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12. Animals were anesthetized with ketamine (35 mg per kilogram of body weight) and xylazine (10 mg per kilogram of body weight) and suspended on an inclined board (80° angle) by their incisors to facilitate instillation of agar beads intratracheally via tracheal cannulation. Beads (150 μl suspended in 0.1 M phosphate and 0.15 M NaCl) containing $4 \times 10^3$ to $10^4$ bacteria were instilled directly via the trachea and animals were returned to their cages. At 24 hours, five randomly selected animals were killed and their lungs were removed and homogenized in sterile tryptic soy broth (TSB) for bacterial enumeration. In all experiments reported, all of the animals

killed at 24 hours had positive lung homogenate cultures containing between 0.2 to 4.2 × $10^5$ colony-forming units (cfu) per lung (mean, 5.1 × $10^4$).

13. Animals were killed by an overdose of carbon dioxide, and the lungs were removed aseptically. Both lobes were placed in 5 ml of TSB in sterile glass homogenizers and mechanically homogenized for 30 s. To quantitate the degree of infection, serial dilutions in sterile saline were made and 0.1-ml amounts were plated onto cetrimide agar or tryptic soy agar (TSA), or both. These plates were incubated overnight at 37°C and mucoid *P. aeruginosa* colonies were enumerated. The remainder of the homogenates in TSB were incubated at 37°C for 5 days, after which they were subcultured onto TSA and cetrimide agar plates. The presence or absence of lung infection was determined by subculture of the total homogenate after the 5 days of incubation and examining these plates for mucoid *P. aeruginosa* colonies.
14. Quantitative counts of the number of bacteria per lung for animals in Table 1 ranged from 0.3 to 25.7 × $10^3$. A statistical analysis based on the quantitative bacterial yields was not performed, since most animals with MEP-specific opsonizing antibody had sterile lung cultures.
15. For mice (virus- and antibody-free, C3H/HeN strain), the challenge inoculum was administered in 50-μl volumes. In all mouse experiments, five animals killed 24 hours after infection resulted in 100% of animals with lung homogenates yielding positive cultures for mucoid *P. aeruginosa* (mean 2.1 × $10^4$ cfu per lung). Mouse lungs were homogenized in 1 ml of TSB, samples were removed for dilutions and enumeration, and TSB was added to achieve a volume of 5 ml. This suspension was incubated for 5 days and subcultured to determine the presence of mucoid *P. aeruginosa*. Quantitative counts ranged from 1.2 to 7.6 × $10^3$ cfu per lung among infected animals.
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# Regulation of Activity of a Transcriptional Anti-Terminator in *E. coli* by Phosphorylation in Vivo

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**Expression of the *bgl* operon of *Escherichia coli* is regulated in vitro by phosphorylation and dephosphorylation of a positive regulatory protein, BglG, which functions in its nonphosphorylated state as a transcriptional antiterminator. The degree of phosphorylation of BglG in vivo was shown to be dependent on the cellular levels of BglF protein, which is both the BglG kinase and phosphatase. The degree of phosphorylation of BglG also depended on the presence or absence of a β-glucoside, the inducer of operon expression. Addition of inducer to cells in growth medium resulted in rapid dephosphorylation of phosphorylated BglG. The *bgl* operon is thus regulated by a sensory system that modulates gene expression by protein phosphorylation and dephosphorylation in response to the external levels of inducer.**

IN THE LAST SEVERAL YEARS IT HAS become clear that protein phosphorylation is as widespread in bacteria as it is in eukaryotes (*1*). However, very few of the phosphorylated bacterial proteins have been characterized. Those that have been characterized include proteins involved in metabolic control, transport processes, bacteriophage infection, and, possibly, DNA replication (*2, 3*). Recent evidence from in vitro studies indicates that transient protein phosphorylation is involved in signal transduction systems that govern chemotaxis, nitrogen assimilation, and osmoregulation. These systems are regulated by pairs of proteins, one acting as sensor, the other as regulator (*4*). Only in the case of chemotaxis and nitrogen assimilation has it been possible to correlate in vitro phosphorylation of the regulatory protein by the sensor with in vivo function. In no case has protein phosphorylation been shown to be involved in regulation of gene expression in vivo.

Recently we reported that reversible protein phosphorylation regulates transcription, in vitro, of the β-glucoside utilization operon in *Escherichia coli* (*5*). This system also contains two regulatory proteins, though these are not members of the groups described above. BglF, a negative regulator of operon expression that also functions as a β-glucoside transporter, is a protein kinase, which, in the absence of β-glucoside inducer, phosphorylates the positive regulatory protein BglG and blocks its function as a transcriptional antiterminator. Upon addition of inducer, BglF dephosphorylates BglG, allowing it to function as a positive regulator of gene expression. On the basis of these studies, we proposed that the BglG protein, which acts as a transcriptional antiterminator (*6, 7*), exists in cells in a phosphorylated, inactive form in the absence of inducer and in a nonphosphorylated, active form in the presence of inducer (*5*).

To confirm that this is true in vivo and thus relevant to *bgl* operon induction, we attempted to show that the two forms of BglG are present in extracts prepared from growing cells. We used two-dimensional (2-D) gel electrophoresis to separate the two protein species from one another and from other proteins in the cell. This procedure involves isoelectric focusing in the first dimension and SDS–polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. The products of an in vitro phosphorylation reaction (*5*) were used to determine the exact location of the two forms of BglG on 2-D gels (*8*). The phosphorylated and nonphosphorylated forms of BglG were separated from one another by this procedure (Fig. 1), the phosphorylated form being more acidic than the nonphosphorylated form. The phosphorylated form of BglG, labeled with [$^{32}$P]phosphate, was detected by autoradiography (Fig. 1A). Protein immunoblot analysis with antiserum to BglG identified both forms of BglG (Fig. 1B). Treatment of the proteins with alkaline phosphatase before gel analysis led to loss of the component identified as phosphorylated BglG, but had no effect on nonphosphorylated BglG (Fig. 1C).

To facilitate in vivo detection of BglG protein, we placed the *bglG* gene under control of the phage T7 promoter, making its expression independent of normal *Bgl* operon expression. Expression of the *bglG* gene was induced from the T7 promoter, in *bgl*$^+$ cells grown in the absence or presence of β-glucoside inducer (salicin). Cells were labeled with [$^{35}$S]methionine, in the presence or absence of rifampicin. Rifampicin prevents labeling of proteins encoded by genes transcribed by *E. coli* RNA polymer-

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