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Production of *Chlamydia pneumoniae* Proteins in *Bacillus subtilis* and Their Use in Characterizing Immune Responses in the Experimental Infection Model

Ulla Airaksinen,¹† Tuula Penttilä,²,³ Eva Wahlström,¹‡ Jenni M. Vuola,¹ Mirja Puolakkainen,²,³ and Matti Sarvas¹*  

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Due to intracellular growth requirements, large-scale cultures of chlamydiae and purification of its proteins are difficult and laborious. To overcome these problems we produced chlamydial proteins in a heterologous host, *Bacillus subtilis*, a gram-positive nonpathogenic bacterium. The genes of *Chlamydia pneumoniae* major outer membrane protein (MOMP), the cysteine-rich outer membrane protein (Omp2), and the heat shock protein (Hsp60) were amplified by PCR, and the PCR products were cloned into expression vectors containing a promoter, a ribosome binding site, and a truncated signal sequence of the α-amylase gene from *Bacillus amyloliquefaciens*. *C. pneumoniae* genes were readily expressed in *B. subtilis* under the control of the α-amylase promoter. The recombinant proteins MOMP and Hsp60 were purified from the bacterial lysate with the aid of the carboxy-terminal histidine hexamer tag by affinity chromatography. The Omp2 was separated as an insoluble fraction after 8 M urea treatment. The purified proteins were successfully used as immunogens and as antigens in serological assays and in a lymphoproliferation test. The Omp2 and Hsp60 antigens were readily recognized by the antibodies appearing after pulmonary infection following intranasal inoculation of *C. pneumoniae* in mice. Also, splenocytes collected from mice immunized with MOMP or Hsp60 proteins proliferated in response to in vitro stimulation with the corresponding proteins.

*Chlamydia pneumoniae* is an important human pathogen that causes acute respiratory infections like pneumonia, bronchitis, and pharyngitis. Furthermore, the association between *C. pneumoniae* and several chronic conditions, including asthma, chronic bronchitis, and atherosclerosis has been investigated by many research groups (9, 10, 19, 32). Antimicrobial therapy, effective in treatment of acute infections, may not be able to resolve the persistent infection associated with the chronic conditions. Therefore, a recent line of research aims at a strategy for preventing or controlling chlamydial infection. Immune intervention could be the means for such a strategy but would require an understanding of the mechanisms of immunity in the various stages of *C. pneumoniae* infection.

Sera from infected individuals recognize several proteins of *C. pneumoniae* (3, 8, 14, 15). One of the best-characterized antigens among different *Chlamydia* species is the major outer membrane protein (MOMP). This 40-kDa protein apparently functions as a porin channel in the outer membrane of *Chlamydia* species (2, 44). Despite the remarkable sequence similarity between the MOMPs of chlamydial species, *C. pneumoniae* MOMP does not seem to be as immunodominant as *Chlamydia trachomatis* MOMP. Another outer membrane protein, Omp2 (62 kDa), has been identified as a target of immune recognition in both *C. trachomatis* and *C. pneumoniae* infections (7, 21, 37). Antibodies against Hsp60 (GroEL) (60 kDa) of *C. trachomatis* have been considered to be important for autoimmune mechanisms in conditions like pelvic inflammatory disease and tubal infertility (6).

For better evaluation the individual *C. pneumoniae* antigens should be obtained free from other *C. pneumoniae* proteins. However, the purification of antigens from *C. pneumoniae* is very difficult, the main obstacle being its pathogenic and parasitic nature, and no host is available for cultivation of *C. pneumoniae* in reasonable quantities. To overcome this, heterologous protein expression systems can be used. *Bacillus subtilis*, a gram-positive, nonpathogenic bacterium, is a very suitable host for production of chlamydial proteins. The widely used laboratory strain 168 contains no innate toxins; in particular, since it is gram positive there is no lipopolysaccharide endotoxin. Its cell wall components are of weak or no biological activity (peptidoglycan and teichoic acid) (12). Effective expression systems for heterologous proteins are available, with the choice of intracellular or secreted mode of production. Fermentation properties of *Bacillus* are favorable, with the feasibility of large-scale cultures. It has been shown that using a *Bacillus* expression vector containing the promoter, a ribosome binding site, and a truncated signal sequence of *Bacillus amyloliquefaciens* α-amylase gene, it is possible to accumulate high levels of intracytoplasmic protein in inclusion bodies (11, 29, 22).

In the present study, we have used a *Bacillus* expression system for the production of *C. pneumoniae* proteins MOMP, Omp2, and Hsp60, and evaluated their immunogenicity in the experimental model for *C. pneumoniae* infection. Experiment-
tal animal models to study C. pneumoniae have been established previously (16, 24, 45). Intranasal inoculation of the bacteria in mice resembles in many respects C. pneumoniae infection in humans: this includes infection kinetics, relatively mild symptoms, the capacity for repeated infections, and the establishment of partial protection. Studies using these models have shown that cell mediated immunity is necessary for protection against C. pneumoniae infection in mice. Specifically, CD8\(^+\) T cells are necessary for protection from both primary infection and reinfection (25, 31).

Here, we show that chlamydial proteins were readily expressed in the Bacillus system as soluble proteins or insoluble inclusion bodies. The inclusion bodies were solubilized with detergents for purification of the recombinant proteins, and the purified proteins were used to produce C. pneumoniae antigen-specific antisera in rabbits.

**MATERIALS AND METHODS**

**Strains and plasmids.** C. pneumoniae Kajaani 6 (K6) was originally provided by Pekka Saikku (National Public Health Institute, Oulu, Finland). Mycoplasma-free C. pneumoniae K6 was used for extraction of the genomic C. pneumoniae DNA for cloning. B. subtilis strains used for expressing chlamydial antigens, and the plasmids used as expression vectors are listed in Table 1.

**Growth conditions.** For the purification of the recombinant proteins, B. subtilis strains expressing chlamydial antigens were grown in shake flasks at 37°C, 300 rpm, in double strength Luria broth (2 \times \) LB) containing 20 g of tryptone, 10 g of yeast extract, and 10 g of NaCl in 1 liter of water. Kanamycin was added to 10 μg/ml when appropriate.

**Plasmids and B. subtilis strains used in this study**

| Plasmid or strain     | Description                                                                 | Source or reference |
|-----------------------|------------------------------------------------------------------------------|---------------------|
| pKTH39                | Cytoplasmic expression vector, amyQ promoter, ribosome binding site and 7 amino-terminal codons of amyQ signal peptide in front of cloning site EcoRI | 22                  |
| pKTH1784              | Like pKTH39; cloning site HindIII                                              | 34                  |
| pKTH3415              | Like pKTH39; cloning site KpnI                                                | This work           |
| pKTH3361              | pKTH39 having C. pneumoniae Hsp60-His\(_6\), encoding insert at EcoRI         | This work           |
| pKTH3391              | pKTH1784 having Omp2-His\(_6\), encoding insert at HindIII                     | This work           |
| pKTH3418              | pKTH3415 having MOMP-His\(_6\), encoding insert at KpnI                      | This work           |

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**Plasmid constructions.** The expression vectors for C. pneumoniae genes contained the promoter and seven N-terminal codons of the signal sequence from B. amyloplaquicis α-amylase gene (amyQ). The expression vectors pKTH39 and pKTH1784 (Table 1), which had the cloning sites EcoRI and HindIII, respectively, were suitable for cloning the C. pneumoniae genes hsp60 and omp2. For the cloning of C. pneumoniae momp, a new cloning site, KpnI, was introduced to the vector pKTH39 by replacing the 0.2-kb ClaI/EcoRI fragment containing the amyQ promoter with a PCR fragment containing the same amyQ promoter and a KpnI site upstream EcoRI (primers 2436 and 61620 [Table 2]). This new expression vector was named pKTH3415. Due to different cloning sites, different extra N-terminal amino acid sequences were introduced to each recombinant protein.

**C. pneumoniae momp** (GenBank accession no. M69230 [27]), omp2 (GenBank accession no. X53511 [40]), and hsp60 (GenBank accession no. M69217 [17]) genes were amplified by PCR from genomic DNA extracted from the K6 isolate. KpnI, HindIII, or EcoRI restriction endonuclease sites were introduced to the primers (Table 2) to enable cloning of momp, omp2, and hsp60, respectively. To facilitate purification of the recombinant proteins, a histidine hexamer tag was introduced to the C termini of the products by the reverse primers. In addition, codons for two glycine residues were added between the chlamydial sequence and the histidine hexamer to minimize a potential disturbance in folding of the newly synthesized protein by the tag. Expand High Fidelity DNA polymerase mix (Roche Biochemicals, Mannheim, Germany) or Dynazyme DNA polymerase (Finnzymes, Helsinki, Finland) was used in PCRs according to the manufacturer’s instructions. The PCR protocol used included 29 cycles (30 s at 94°C, 20 s at 50°C, and 1 min 30 s at 72°C) preceded by 4 min of predenaturation at 94°C and followed by a 7-min extension at 72°C after the cycles in the Programmable Thermal Controller (MJ Research, Inc.). The PCR products were purified using...
a QiaQuick PCR purification kit (Qiagen Inc., Hilden, Germany) before cloning into the expression vectors. B. subtilis was transformed using the method of Czyczek et al. (5). The transformants obtained were screened by PCR or by restriction enzyme analysis. Also, the presence of correct inserts was confirmed by DNA sequencing (M. Rottenberg, Karolinska Institutet, Stockholm, Sweden).

**Western blotting.** Expression of the recombinant C. pneumoniae proteins in B. subtilis was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Cells of 1 ml of bacterial mid-log culture were pelleted and resuspended in 100 μl of protoplast buffer (20 mM potassium phosphate [pH 7.5 to 8], 15 mM MgCl₂, 20% sucrose and lysisomce [1 mg/ml]) for 30 min at 37°C. Lysed bacteria were boiled in sample buffer, and the proteins were separated by SDS-9% PAGE according to the method of Laemmli (20). The separated proteins were transferred to polypeptided difluoride membrane (Immobilon P, Millipore, Bedford, Mass.).

**Immunoblotting.** Expression of purified C. pneumoniae EBs was done as described previously (30) with some modifications. Briefly, the lysates of purified EBs were electrophoresed in SDS-PAGE. After electrophoresis, separated proteins were fixed using the method of O’O’Brien-Harris, anti-Omp2-Hiis, or anti-Hsp60-Hiis rabbit sera (1:500) overnight. After washing the filters were incubated with swine anti-rabbit peroxidase-conjugated immunoglobulins (1:3000) (Dako, Glostrup, Denmark), and the color was developed using 4-chloro-1-naphtol as substrate.

**Production of rabbit antisera.** Rabbits were immunized subcutaneously three times with 50 μg of each protein: a first injection with Freund’s complete adjuvant, a second injection at day 14 with incomplete Freund’s adjuvant, and a third at day 42 with incomplete Freund’s adjuvant. Sera were collected 10 days after the third injection. The rabbit sera were named as follows: KH1505 (α-Hsp60-Hiis), KH1508 (α-MOMP-Hiis), and KH1510 (α-Omp2-Hiis).

**Microimmunofluorescence assays (MIF).** MIF were performed as described in reference 38 using C. pneumoniae K6 EBs as antigens. Briefly, the test uses purified and formalin-fixed chlamydial EBs as antigen. Serum samples containing antichlamydial antibodies were added on EB-coated slides for 30 min at 37°C. Slides were washed with phosphate-buffered saline (PBS), and fluorescein isothiocyanate conjugates were added on slides for 30 min at 37°C. After washing with PBS, slides were mounted and viewed under fluorescence microscope.

**Mouse infection experiments.** Specific-pathogen-free female BALB/c mice (Bromborough Breeding and Research Centre Ltd., Ry, Denmark) kept in ventilated containers and given food and water ad libitum, were used at 6 to 8 weeks of age. Infection of mice with 10⁶ inclusion forming units (IFU) of C. pneumoniae K6 isolate was done intranasally as described previously (24). The sera of four to six mice were collected at days 2, 6, and 17 after infection. Mice were rechallenged 8 weeks after the first challenge with the same amount of C. pneumoniae and again sera of infected mice were collected at days 2, 7, and 11 after the rechallenge.

**Mouse immunization.** Antigens used to immunize mice were cultivated stock of C. pneumoniae K6 isolate boiled for 10 min or C. pneumoniae recombinant proteins produced in Bacillus. They were either boiled (Hsp60-Hiis) or used as such (MOMP-Hiis). Boiled mock sample, prepared similarly to the infectious stock but containing no C. pneumoniae, was used as a control in the immunizations. Groups of 12 mice were immunized intraperitoneally (i.p.) twice with 10 days interval, with approximately 10 μg of EB protein (~10⁹ IFU) or with 100 μg of recombinant proteins. After the immunizations, two mice were bled for antibody measurements, their spleens were dissected, and a single-cell suspension was prepared for the proliferation assays. The rest of the mice were challenged with live C. pneumoniae 11 days after the second immunization, and sera were obtained from individual mice for antibody measurements at days 4 and 10 after the challenge.

**Antibody ELISA.** ELISA was done as described earlier by Penttilä et al. (26). In brief, polystyrene 96-well plates (Nalge Ltd., Hereford, United Kingdom) were coated with heterologously expressed C. pneumoniae proteins (0.75 μg/ml) overnight at room temperature. After blocking with 5% bovine serum albumin in PBS (1 h, 37°C), serially diluted mouse sera were allowed to bind to the proteins 1 h at room temperature. The plates were washed with PBS~0.05% Tween 20, and the binding was detected with horseradish peroxidase-labeled antibody to mouse immunoglobulins (Dako A/S, Denmark). After washing, the substrate (BM Blue POD substrate; Roche Diagnostics GmbH) was added and the abs absorbance measured at 450 nm. The ELISA titers were expressed as logarithmic values (log₁₀) that represent the inverse values of mean end point dilutions of sera read at an optical density of 0.3 (18).

**Lymphoproliferation assay.** Single-cell suspensions of splenocytes were prepared by mechanical homogenization of spleens and lysis of erythrocytes with short hypotonic shock with H₂O. Cells from two spleens were pooled and resuspended in complete growth medium containing RPMI 1640 (Sigma, St. Louis, Mo.), 10% fetal calf serum, 10 μM HEPES (Sigma), L-glutamine (0.3 mg/ml; Gibco BRL, Life Technologies, Paisley, Scotland), nonessential amino acids (10 mM) and L-glutamine (10 μM/liter), streptomycin (10 μg/ml/liter), and 50 μM 2-mercaptoethanol (Sigma). The proliferative response of 0.2 × 10⁵ isolated splenocytes to a 5-μg/ml concentration of Hsp60-Hiis, Omp2-Hiis, and MOMP-Hiis, and purified formalin-inactivated C. pneumoniae EBs (1 μg/ml) was detected in a 3-day proliferation assay using [H]-labeled thymidine (Amersham, Aylesbury, United Kingdom) similarly to that described by Penttilä et al. (24). The proliferative response was expressed as percentage counts per minute (cpm) of the carrier-induced proliferation – background proliferation/ background proliferation. The mean background proliferation (without stimulants) was 2966 cpm.

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Statistics. Nonparametric Mann-Whitney U test was used for statistical comparison of the groups.

The Institutional Ethics Committee on Animal Experimentation of National Public Health Institute and the provincial state of southern Finland approved all the animal experiments. When genetically modified organisms were used we followed the safeguards and the procedure of notification to the Finnish Board on Gene Technology as obliged by the Finnish law on Gene Technology.

RESULTS

Plasmids and expression strains for production of C. pneumoniae proteins in B. subtilis. Expression of the chlamydial proteins in B. subtilis was achieved using a multicopy plasmid vector system designed for intracellular expression (23). The expression system is based on pUB110 derivatives carrying the B. amyloliquefaciens/H9251-amylose promoter and a partial signal sequence encoding seven amino acids. Expression from these plasmids is constitutive, and the partial signal sequence was retained to ensure efficient translation of the heterologous genes inserted downstream. Two or four additional N-terminal amino acids were introduced to the cloning sites, different in each vector (Fig. 1). The C-terminal end of the produced chlamydial proteins was extended with a histidine hexamer with a linker of a glycine dimer. The recombinant plasmids were transferred for expression into B. subtilis WB600, which is devoid of six extracellular proteases, or into IH6140, with a low level of exoproteases (Table 1).

The hsp60 gene (nucleotides 617 to 2248 of GenBank sequence accession no. M69217) was amplified by PCR, and the PCR product was inserted at the EcoRI site of pKTH39 to give pKTH3361 (Fig. 1). B. subtilis WB600 was transformed with the expression plasmid resulting the strain IH7115. DNA sequencing of the plasmid showed all together three unintended nucleotide changes resulting in amino acid changes—G 1133 A 1133 (Gly 3 Arg), A 1896 G 1896 (Glu 3 Gly), and CG 2145 GC 2145 (Arg 3 Ala)—and, furthermore, one silent change G 2119 A 2119. The numbers refer to the M69217 DNA sequence.

The momp gene (nucleotides 386 to 1483 of the DNA sequence; GenBank accession no. M69230) was amplified by PCR to give a product in which the codons for the 23 N-terminal amino acids were omitted. The momp-His6 PCR fragment was inserted at the KpnI site of pKTH3415, resulting in pKTH3418 (Fig. 1).

Puriﬁcation of the heterologous proteins. B. subtilis cells were disrupted with lysozyme and centrifuged. MOMP-His6 and Omp2-His6 were found in the particular fraction as inclusion bodies, while Hsp60-His6 was mainly in the soluble fraction. However, a fraction of Hsp60-His6 remained insoluble under the conditions used, and no attempts to recover this fraction was made. The soluble form of the Hsp60-His6 was bound to the Ni-NTA resin with about 50% efficiency and eluted from the column with imidazole of low concentration.
(100 mM). Eluted Hsp60-His<sub>6</sub> was found to migrate in two bands, 62 and 60 kDa, respectively, in SDS-PAGE. During dialysis the proportion of the 60-kDa variant increased, indicating degradation. Both 62- and 60-kDa proteins that were seen in CBB-stained SDS-polyacrylamide gels (Fig. 2A) reacted with rabbit anti-Cpn serum KH1500 and also with monoclonal anti-His<sub>6</sub> antibody and Ni-NTA conjugate (data not shown).

The MOMP-His<sub>6</sub> was produced as insoluble inclusion bodies in <i>B. subtilis</i>. MOMP-His<sub>6</sub> was solubilized with urea before binding to Ni-NTA resin, and it was eluted under denaturing conditions with 250 mM imidazole in the presence of 8 M urea. β-Mercaptoethanol was included to prevent the formation of intermolecular disulfide bonds. Eluate was precipitated by dialyzing against buffer devoid of urea. The precipitate was recovered from the dialysate by pelleting at 20,000 × g and by dissolving the pellet in 1% SDS and 2 mM DTT. The purified MOMP-His<sub>6</sub> ran as one band of 40 kDa in SDS-PAGE (Fig. 2B) and was recognized by rabbit anti-Cpn serum (KH1500) and Ni-NTA conjugate (data not shown).

The Omp2-His<sub>6</sub> was also produced as insoluble inclusion bodies in <i>B. subtilis</i>. In contrast to MOMP-His<sub>6</sub>, this protein was not soluble in urea and thus could not be purified by the Ni-NTA affinity method. However, after the solubilization with 8 M urea, the insoluble fraction was found to be a rather pure preparation of Omp2-His<sub>6</sub> based on SDS-PAGE analysis. Essentially pure protein was obtained in milligram quantities. A major 60-kDa protein as well as some minor forms of higher molecular mass was recognized by CBB staining (Fig. 2C) as well as in Western blotting by rabbit anti-<i>C. pneumoniae</i> EB antiserum (KH1500) and the conjugate reacting with the His tag (data not shown).

**Immune sera from rabbits.** The antisera prepared by immunizing rabbits with the purified protein preparations reacted in Western blotting at high dilution with the SDS-PAGE-separated <i>C. pneumoniae</i> EBs as expected: α-Hsp60-His<sub>6</sub> and α-Omp2-His<sub>6</sub> sera recognized proteins of approximately 60 kDa, and α-MOMP-His<sub>6</sub> serum recognized a protein of approximately 43 kDa (Fig. 3). In MIF, where formalin-inacti-
however, a response against Omp2-His₉ protein (mean titer 3.3) was detected (Fig. 4). The strongest reactivity with Omp2-His₉ was seen approximately 4 weeks after primary infection, and the antibody levels of infected mice stayed high at least 6 weeks (data not shown). After rechallenge there was a rapid antibody response against MOMP and Hsp60 (the change of mean titers from 2.3 to 2.8 and from 2.2 to 3.8, respectively), indicating that mice had developed immunological memory also against these antigens. The response against Omp2-His₉ protein was enhanced (mean titers > 4).

Immunization i.p. with two doses of heat-killed EBs of *C. pneumoniae* and challenge after immunizations with 10⁶ IFU of *C. pneumoniae* induced a rapid and strong antibody response in the mice against MOMP and Omp2 (mean titers, 3.3 and 3.3, respectively), but no anti-Hsp60 response (mean titer, 1.7) could be detected by EIA. The mice that were not immunized developed an antibody response against the proteins more slowly, a response similar to that observed in primary *C. pneumoniae* infection (Fig. 5).

**Utilization of *C. pneumoniae* proteins in the lymphoproliferation assay.** When used as reagents to study cell mediated immunity the denatured conformation of heterologous proteins is of minor significance. Instead, a low background proliferative response, which indicates the reagents to be devoid of specific stimulatory effects, is important.

BALB/c mice were immunized with heat-killed *C. pneumoniae*, MOMP-His₉, or heat-aggregated Hsp60-His₉, after which the induction of proliferative responses of splenocytes was tested. Whereas the proliferative indices of splenocytes from mock-immunized mice against *C. pneumoniae* EBs, MOMP-His₉, and Hsp60-His₉, were below 2 (i.e., background proliferative response) the proliferative response against *C. pneumoniae* EBs was increased five to sevenfold in the three immunization groups. Increased proliferative responses against the corresponding proteins were detected in mice immunized with MOMP or Hsp60 proteins. Mice immunized with heat-killed *C. pneumoniae* showed a moderately increased proliferative response against Hsp60 but not against MOMP (Fig. 6).

Omp2-His₉ proteins were not used in mouse immunizations but in a separate set of experiments the protein was shown to induce a background proliferative response of 2.05 ± 0.49 in control splenocytes (data not shown).

**DISCUSSION**

*Bacillus subtilis* is an excellent host for expression and production for heterologous proteins for immunological studies. Here, we describe expression of three *C. pneumoniae* proteins (MOMP, Omp2, and Hsp60) in *B. subtilis*. The proteins could be obtained in essentially pure form and in large quantities. The Hsp60 protein was soluble with native conformation, and the outer membrane proteins of *C. pneumoniae* (MOMP and Omp2) were at the denatured state. They were found in *B. subtilis* as inclusion bodies like most membrane proteins produced at high level in heterologous hosts, and they could be solubilized and purified only under strongly denaturing conditions. The denatured form sets constraints in terms of the presence of antigenic epitopes found in native proteins. It is probable that during the microbial infection the range of antibodies covers both native and denatured epitopes of the microbial antigens, although many epitopes exposed on the bacterial surface are discontinuous, and the detection of antibodies raised to these epitopes requires antigens of native conformation. On the other hand, when cell-mediated immunity is studied the conformation of the proteins used as reagents is of minor significance. For such studies it is of special significance that proteins produced in the *Bacillus* expression system be free of endotoxin and consequently devoid of unspecific stimulatory effects in immunological studies. In this study, we evaluated their use as immunogens and as tools to study humoral and cell-mediated immunity during experimental *C. pneumoniae* infection.

The *B. subtilis*-produced chlamydial proteins were used to produce polyclonal, monospecific antisera. By Western blotting, the antisera from rabbits immunized with MOMP-His₉, Omp2-His₉ or Hsp60-His₉ proteins specifically recognized pro-
proteins of the corresponding size in purified \emph{C. pneumoniae} EB. However, in the MIF test, where antibodies react with as-yet-undefined antigens present on the surface of whole \emph{C. pneumoniae} organisms, no reactivity was seen with any of the antisera. This is not surprising, since the rabbit antisera were likely to recognize only linear epitopes of Omp2 and MOMP, as the animals were immunized with denatured Omp2-His\textsubscript{6} and MOMP-His\textsubscript{6} proteins. Both Omp2 and MOMP are part of the sarcosyl-insoluble fraction of \emph{C. pneumoniae} EBs, the outer membrane complex (28). Earlier, MOMP was not considered

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\includegraphics[width=\textwidth]{fig5}
\caption{Antibody response induced in BALB/c mice against recombinant MOMP-His\textsubscript{6}, Omp2-His\textsubscript{6}, and Hsp60-His\textsubscript{6} proteins after immunization of mice with heat-aggregated \emph{C. pneumoniae} EBs. Mice were immunized i.p. twice with 100 \textmu{g} of EBs. Ten days after the last immunization mice were challenged with \emph{C. pneumoniae}, and antibodies were measured by EIA 0, 4, and 10 days after challenge. The EIA titers were expressed as logarithmic (log\textsubscript{10}) value of titers, which represent the inverse values of mean end point dilutions of sera (error bar, range) read at an optical density of 0.3. *, \(P < 0.05\) obtained in statistical analysis between immunized and control groups of mice (= mock immunized).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6}
\caption{\emph{C. pneumoniae} MOMP-His\textsubscript{6} (white bars) and Hsp60-His\textsubscript{6} (gray bars) proteins produced in \emph{Bacillus} were used as antigens in an in vitro proliferation assay in parallel with formalin-inactivated \emph{C. pneumoniae} EBs (black bars). Mice were immunized at days 0 and 10, and splenocytes were isolated at day 21. Isolated splenocytes were stimulated with antigens or medium alone (background proliferation) for 3 days, and the proliferation was detected as incorporation of \[^{3}H\]thymidine during the last 16 to 18 h of incubation. Proliferation index was calculated as follows: (antigen induced proliferation − background proliferation)/background proliferation. nd, not done.}
\end{figure}
to be surface exposed, because in immunoblotting human sera did not recognize MOMP (3) and because a monoclonal antibody against C. pneumoniae MOMP failed to react with purified EBs in immunoelectron microscopy (4). Recently, however, it was suggested that MOMP of C. pneumoniae is exposed on the surface of the bacteria, but antibodies recognize a conformational epitope of MOMP, and could thus not be detected with sera raised against denatured protein (42). That is also the case with other porin proteins like the mingenococcal PorA (22). Although Omp2 is a target of immune recognition during chlamydial infections (37, 8), it has not been detected on the surface of C. pneumoniae EBs (41), whereas the amino-terminal part of the corresponding C. trachomatis protein is surface exposed (35). This limited exposure of the protein on the surface might explain why antibodies against the whole chlamydial Omp2 protein do not react with intact EBs, whereas an antibody against that specific exposed peptide is able to bind to EBs (35). Hsp60 is a cytoplasmic protein, which is also not likely to be exposed on EBs.

Definite serological diagnosis of chlamydial infections requires the use of MIF method (38, 39). However, the method is technically demanding, requires expertise in interpretation, and is not widely available. We evaluated whether the produced proteins could be used as antigens in serological EIA. During experimental C. pneumoniae infection in mice, a strong antibody response against the Omp2-His6 protein appeared already after primary infection. This is in accordance with the earlier studies showing that Omp2, even in denatured form, is a major immunogen recognized during human C. pneumoniae infection (21, 7). In mice, antibody response against MOMP-His6 and Hsp60-His6 protein was negligible after primary infection (21, 7). Antibodies induced by chlamydial infection may recognize both conformational and linear epitopes of the antigen, and the former ones may not react with the recombinant proteins. During human chlamydial infection, serum antibody response against the Hsp60-His6 (13) and Omp2-His6 proteins (our unpublished observations) could be detected by EIA suggesting that the proteins could be useful for measuring antigen-specific responses also in human sera.

The chlamydial proteins were produced in B. subtilis, a gram-positive bacterium that contains no lipopolysaccharide (endotoxin). Also, its teichoic acid has weak or no biological activity (12). When used as stimulatory proteins in lymphoproliferation assay, the purified protein preparations induced minimal mitogenic activity as shown by relatively low background levels observed in proliferative response of splenocytes from naive or mock-immunized mice. Furthermore, when mice where immunized with the proteins or heat-killed whole bacteria, a clear increment was detected in proliferative responses against the corresponding proteins. Increased proliferative response against MOMP was also observed after DNA immunization (26). This suggests that the recombinant proteins are useful reagents when studying cellular immune responses. The denatured state of the outer membrane proteins (MOMP and Omp2) is of less significance in these assays. Also, the proteins described here, like whole bacteria, have been shown to induce human monocyte-derived macrophages to secrete the 92-kDa gelatinase (36).

In this study, we showed that B. subtilis is a suitable host for production of chlamydial proteins. The proteins were easily expressed, could be purified, were suitable for production of antisera, and could successfully be used as reagents to study humoral and cellular immunity during experimental C. pneumoniae infection.

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AUTHOR’S CORRECTION

Production of *Chlamydia pneumoniae* Proteins in *Bacillus subtilis* and Their Use in Characterizing Immune Responses in the Experimental Infection Model

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