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

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Received 19 April 2002/Returned for modification 20 November 2002/Accepted 18 January 2002

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 infections. 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, 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 MOMP s 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. Experimen-
Mycoplasma free strains expressing chlamydial antigens were grown in shake flasks at 37°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 MinPrep kit (Promega, Madison, WI). Double-stranded DNA fragments were ligated into the expression vectors pKTH39 and pKTH3415 to create the recombinant plasmids pKTH3418 and pKTH3414. These plasmids were used to transform B. subtilis IH7115, a strain carrying a plasmid with a chloramphenicol resistance gene, to obtain stable transformants. The DNA sequences of these plasmids were confirmed by DNA sequencing.

### MATERIALS AND METHODS

#### Strains and plasmids

*C. pneumoniae* 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 DNA. *C. pneumoniae* DNA was isolated by the phenol-chloroform method. Strains and 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, 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 MinPrep kit (Promega, Madison, WI). Double-stranded DNA fragments were ligated into the expression vectors pKTH39 and pKTH3415 to create the recombinant plasmids pKTH3418 and pKTH3414. These plasmids were used to transform *B. subtilis* IH7115, a strain carrying a plasmid with a chloramphenicol resistance gene, to obtain stable transformants. The DNA sequences of these plasmids were confirmed by DNA sequencing.

### Table 1: Plasmids and *B. subtilis* strains used in this study

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKTH39</td>
<td>Cytoplasmic expression vector, <em>amyQ</em> promoter, ribosome binding site and 7 amino-terminal codons of <em>amyQ</em> signal peptide in front of cloning site <em>EcoRI</em></td>
<td>22</td>
</tr>
<tr>
<td>pKTH1784</td>
<td>Like pKTH39; cloning site <em>HindIII</em></td>
<td>34</td>
</tr>
<tr>
<td>pKTH3415</td>
<td>Like pKTH39; cloning site <em>KpnI</em></td>
<td>This work</td>
</tr>
<tr>
<td>pKTH3361</td>
<td>pKTH39 having <em>C. pneumoniae</em> Hsp60-His6 encoding insert at <em>EcoRI</em></td>
<td>This work</td>
</tr>
<tr>
<td>pKTH3391</td>
<td>pKTH1784 having Omp2-His6 encoding insert at <em>HindIII</em></td>
<td>This work</td>
</tr>
<tr>
<td>pKTH3418</td>
<td>pKTH3415 having MOMP-His6 encoding insert at <em>KpnI</em></td>
<td>This work</td>
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**Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IH6140</td>
<td><em>B. subtilis</em> IA289; NNG mutagenized; reduction of total protease</td>
<td>33</td>
</tr>
<tr>
<td>WB600</td>
<td><em>B. subtilis</em> WB600 (<em>trpC2</em>Delta<em>prE</em>Delta<em>prE</em>Delta<em>hpD</em>Delta*prB)</td>
<td>43</td>
</tr>
<tr>
<td>168</td>
<td><em>B. subtilis</em> <em>trpC2</em></td>
<td>1</td>
</tr>
<tr>
<td>IH7339</td>
<td><em>B. subtilis</em> 168 carrying pKTH3415</td>
<td>This work</td>
</tr>
<tr>
<td>IH7115</td>
<td><em>B. subtilis</em> WB600 carrying pKTH3361</td>
<td>This work</td>
</tr>
<tr>
<td>IH7279</td>
<td><em>B. subtilis</em> IH6140 carrying pKTH3391</td>
<td>This work</td>
</tr>
<tr>
<td>IH7342</td>
<td><em>B. subtilis</em> WB600 carrying pKTH3418</td>
<td>This work</td>
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### Table 2: PCR primers used for cloning to introduce a novel cloning site into *C. pneumoniae* genes

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Orientation</th>
<th>Sequence</th>
<th>Target</th>
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<tbody>
<tr>
<td>2436</td>
<td>F</td>
<td>AACCGGATCCATCGATGTGTTAGAAAG</td>
<td><em>amyQ</em></td>
</tr>
<tr>
<td>61620</td>
<td>R</td>
<td>CGGAATTCGCTACCGCTTCTTCTGAAA</td>
<td><em>amyQ</em></td>
</tr>
<tr>
<td>2955</td>
<td>F</td>
<td>GGAATTCGATGCGGCAAGGAAAT</td>
<td><em>hsp60</em></td>
</tr>
<tr>
<td>6658</td>
<td>R</td>
<td>GGAATTCGATGCGGCAAGGAAAT</td>
<td><em>momp</em></td>
</tr>
<tr>
<td>61619</td>
<td>F</td>
<td>CGGTATACCGTCTGTTAGGAAAC</td>
<td><em>momp</em></td>
</tr>
<tr>
<td>61632</td>
<td>R</td>
<td>CGGTATACCGTCTGTTAGGAAAC</td>
<td><em>momp</em></td>
</tr>
<tr>
<td>616600</td>
<td>F</td>
<td>CGGTATACCGTCTGTTAGGAAAC</td>
<td><em>momp</em></td>
</tr>
<tr>
<td>616599</td>
<td>R</td>
<td>CGGTATACCGTCTGTTAGGAAAC</td>
<td><em>momp</em></td>
</tr>
</tbody>
</table>

* F, forward; R, reverse.
a QiAQuick PCR purification kit (Qiagen Inc., Hilden, Germany) before cloning into the expression vectors. *B. subtilis* was transformed using the method of Czyczan 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 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 polybuffering (20 mM NaOAc, pH 7.2, containing 20 mM EDTA and 50 mM DTT). The cells were then sonicated for 1 min and incubated for 30 min at 37°C with shaking after which 30 ml of urea was added to final 8 M concentration. After sonication nonsoluble proteins were pelleted by centrifugation at 20,000 × g and the pellet was solubilized in 30 ml of buffer C containing 0.5% SDS and 5 mM DTT. The amount of recombinant proteins was estimated by SDS-PAGE followed by CBB staining.

**Production of rabbit antiserum.** 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-His6), KH1508 (α-MOMP-His6), and KH1510 (α-Omp2-His6).

Microimmunofluorescence assays (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 (Bomholtgard 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 10° 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 challenged 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 cultured stock of *C. pneumoniae* K6 isolate boiled for 10 min or *C. pneumoniae* recombinant proteins produced in *B. subtilis*. They were either boiled (Hsp60-His6) or used as such (MOMP-His6). 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 inoculated intraperitoneally (i.p.) twice with 10 days interval, with approximately 10 μg of EB protein (~10° 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 EIA.** EIA was done as described earlier by Penttila 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 G (Dako A/S, Denmark). After washing, the substrate (BM Blue POD substrate; Roche Diagnostics GmbH) was added and the absorbance measured at 450 nm. The EIA titers were expressed as logarithmic (log10) values 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 mM HEPES (Sigma), 1-glutamine (0.3 mg/ml; Gibco BRL, Life Technologies, Paisley, Scotland, United Kingdom), 50 μg/ml streptomycin (10 U/ml; Sigma), 100 μg/ml penicillin G, 10 mM 2-mercaptoethanol (Sigma). The proliferative response of 0.2 × 10^6 isolated splenocytes to a 5-μg/ml concentration of Hsp60-His6, Omp2-His6, and MOMP-His6, and purified formyl- and 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 Penttila et al. (24). The proliferative response was expressed as percentage of the total counts of the spleen of the same animals that received no antigen and background proliferation. The mean background proliferation (without stimulants) was 2.966 cpm.
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-amylase 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 in strain IH7115. DNA sequencing of the plasmid showed all together three unintended nucleotide changes resulting in amino acid changes—G1133→A1133 (Gly→Arg), A1896→G1896 (Glu→Gly), and CG2145→GC2145 (Arg→Ala)—and, furthermore, one silent change G2119→A2119. 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).

The coding region for Omp2 (nucleotides 805 to 2406; GenBank accession no. X53511), excluding the codons for the 22 N-terminal amino acids, was amplified by PCR. The PCR product was ligated at the HindIII site of pKTH1784, resulting in pKTH3391 (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 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.

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.
Eluted Hsp60-His6 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-His6 antibody and Ni-NTA conjugate (data not shown).

The MOMP-His6 was produced as insoluble inclusion bodies in B. subtilis. MOMP-His6 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-His6 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-His6 was also produced as insoluble inclusion bodies in B. subtilis. In contrast to MOMP-His6, 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-His6 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-C. pneumoniae 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 C. pneumoniae EBs as expected: α-Hsp60-His6 and α-Omp2-His6 sera recognized proteins of approximately 60 kDa, and α-MOMP-His6 serum recognized a protein of approximately 43 kDa (Fig. 3). In MIF, where formalin-inactivated whole C. pneumoniae EBs were used as antigen, no reactivity was seen (data not shown).

**Utilization of heterologous proteins (MOMP-His6, Omp2-His6, and Hsp60-His6) as EIA antigens.** The significance for antigenicity of the denatured state of proteins MOMP and Omp2 prepared from B. subtilis was studied. Mice were infected either with living C. pneumoniae bacteria (proteins and epitopes in natural conformation) or immunized with heat-killed bacteria (proteins and epitopes presumably in denatured conformation) to evaluate the possible differences in the specificity of the immune sera against heterologous proteins.

Mice infected intranasally with living C. pneumoniae did not produce antibodies against the MOMP-His6 or Hsp60-His6 proteins (mean titers <2.0) in 2 weeks after the challenge;
however, a response against Omp2-His₆ protein (mean titer 3.3) was detected (Fig. 4). The strongest reactivity with Omp2-His₆ 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-His₆ 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 unspecified stimulatory effects, is important.

BALB/c mice were immunized with heat-killed C. pneumoniae, MOMP-His₆, or heat-aggregated Hsp60-His₆, 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-His₆ and Hsp60-His₆, 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-His₆ 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-His₆, Omp2-His₆ or Hsp60-His₆ proteins specifically recognized pro-
proteins of the corresponding size in purified C. pneumoniae EB. However, in the MIF test, where antibodies react with as-yet-undefined antigens present on the surface of whole 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-His6 and MOMP-His6 proteins. Both Omp2 and MOMP are part of the sarcosyl-insoluble fraction of C. pneumoniae EBs, the outer membrane complex (28). Earlier, MOMP was not considered

FIG. 5. Antibody response induced in BALB/c mice against recombinant MOMP-His6, Omp2-His6, and Hsp60-His6 proteins after immunization of mice with heat-aggregated C. pneumoniae EBs. Mice were immunized i.p. twice with 100 μg of EBs. Ten days after the last immunization mice were challenged with C. pneumoniae, and antibodies were measured by EIA 0, 4, and 10 days after challenge. The EIA titers were expressed as logarithmic (log10) 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 of <0.05 obtained in statistical analysis between immunized and control groups of mice (= mock immunized).

FIG. 6. C. pneumoniae MOMP-His6 (white bars) and Hsp60-His6 (gray bars) proteins produced in Bacillus were used as antigens in an in vitro proliferation assay in parallel with formalin-inactivated 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 [3H]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.
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 meningococcal 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, but the rapid appearance of these antibodies after the second infection suggests that the mice had developed immunological memory against these proteins during the first infection. Similarly, we have earlier shown that after intramuscular immunization with DNA coding for C. pneumoniae Omp2, the mice developed antibodies against Omp2-His6, whereas development of antibodies against Hsp60-His6 required the immunization with the corresponding DNA plasmid and the challenge (26). However, mice immunized with denatured (heat-killed) C. pneumoniae and, as mentioned in the discussion earlier, rabbits immunized with the recombinant proteins did develop a strong antibody response against MOMP-His6 proteins. This suggests the correct conformation of the MOMP antigen is critical for accurate detection of the antibody response. Antibodies induced by chlamydial infection may recognize both conformational and linear epitopes of the antigens, 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 ELISA 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 naïve 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.

ACKNOWLEDGMENTS

This work was supported by contract B104-CT96-0152 of the Biotechnology Programme of the Commission of the European Union.

We acknowledge Lynda Jackson for her contribution to construct the Hsp60 recombinants and Leena Liesirova, Outi Rautio, Irene Virmikangas, Satja Kokkoneni, and Anja Rattuhiinen for skillful technical assistance. We thank P. Helena Makela for her valuable contribution to the writing of this paper and Juha Heikkiä, University of Lapland, Rovaniemti, Finland, for help with statistical analysis.

REFERENCES


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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Volume 10, no. 3, p. 367–375, 2003. The article byline should read as given above.

Page 367: The following footnote should be added: “§Present address: Via Guiseppe Motta 34, CH 6900 Lugano, Ticino, Switzerland.”

Page 374: The Acknowledgments section should appear as shown below.

This work was supported by contracts BIO4-CT96-0152 of the Biotechnology Program, and ERBCHGC930360 of the Capital and Mobility Program (L.J.) of the Commission of the European Union.

We acknowledge Leena Liesirova, Outi Rautio, Irene Viinikangas, Saija Kokkoneni, and Anja Ratilainen for skillful technical assistance.

We thank P. Helena Mäkelä for her valuable contribution to the writing of this paper and Juha Heikkilä, University of Lapland, Rovaniemi, Finland, for help with statistical analysis.