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Cloning and Sequencing of the Genes Coding for the 10- and 60-kDa Heat Shock Proteins from *Pseudomonas aeruginosa* and Mapping of a Species-Specific Epitope

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A genomic library of *Pseudomonas aeruginosa* DNA was screened with a monoclonal antibody (MAb 2528) specific for the *P. aeruginosa* 60-kDa heat shock protein. A positive clone, pAS-1, was isolated. The gene coding for *P. aeruginosa* chaperonin (hsp60) was localized to a 2-kb *Eco*RI fragment subcloned in pAS-2. A sequence analysis of pAS-2 and parts of pAS-1 identified two open reading frames that encoded proteins with calculated molecular masses of 10 and 57 kDa. In amino acid sequence comparison studies the sequences of these proteins, which were designated GroES and GroEL, exhibited up to 78% homology with known prokaryotic sequences of 10- and 60-kDa heat shock proteins (hsp10 and hsp60). In order to map the epitope recognized by MAb 2528, a series of GroEL nested carboxy-terminal deletion clones were tested with MAb 2528. We identified the clone with the shortest insertion that was still recognized by MAb 2528 and the clone with the largest insertion that was not recognized by MAb 2528. The 3′ ends of the insertions were determined by sequencing and were found to delimit a region that encoded 25 amino acid residues. Synthetic oligonucleotides that coded for peptides possibly resembling the epitope within this region were ligated into expression vector pGEX-3X, and fusion proteins expressed by these clones were tested for reactivity with MAb 2528. By using this method we determined that the decapeptide QADIEARVLQ (positions 339 to 348 on GroEL) was responsible for the binding of *P. aeruginosa*-specific MAb 2528.

Pseudomonas aeruginosa is one of the most important causes of invasive infections in compromised patients with serious underlying diseases, such as extensive burns, leukemia, or cystic fibrosis (23). On the other hand, this organism is often isolated from patients in intensive care units or surgical wards who do not have clinical signs and symptoms of infection (20).

In studies on the antibody response to *P. aeruginosa*, Hoiby described a "common antigen" which exhibited cross-reactivity with a wide spectrum of gram-negative bacteria (12). This immunodominant protein antigen was isolated and biochemically characterized by Sompolinsky et al. (29, 30). Cross-reactivity between the common antigen of *P. aeruginosa* and *Legionella pneumophila* has been shown to hamper the specificity of serological tests for members of the family *Legionellaceae* (4) and seems to be the main cause of false-positive results for *Legionella* serology in patients with cystic fibrosis (36).

Later, the common antigen turned out to be one of the 60-kDa heat shock or stress proteins, a group of highly immunoreactive proteins that includes species- and genus-specific epitopes in addition to broadly cross-reactive epitopes (27). These highly conserved proteins are among the most abundant proteins found not only in bacteria but also in plant chloroplasts and mitochondria (13, 22). They have been shown to assist the posttranslational assembly of oligomeric protein structures and therefore are called molecular chaperones or chaperonins (9). Their important role as main targets of the immune response and the accumulating evidence for the involvement of these molecules in autoimmune processes have been reviewed recently by Kaufmann (14) and Young and Elliott (37).

In this study we used a monoclonal antibody raised against *P. aeruginosa* which recognizes a species-specific epitope on the 60-kDa heat shock protein or common antigen. We cloned and sequenced two genes which represent one operon that codes for the 10- and 60-kDa heat shock proteins of *P. aeruginosa*. In an attempt to define suitable peptides for species-specific serological tests, we then mapped the epitope for the *P. aeruginosa*-specific monoclonal antibody on the 60-kDa heat shock protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* ATCC 27853 was obtained from the American Type Culture Collection. Host strain *Escherichia coli* DH5α was purchased from GIBCO BRL, Eggenstein, Federal Republic of Germany. *E. coli* BW313, a host strain containing no amber suppressor, has been described previously by Kunkel et al. (15). Plasmid vector pEMBL8 has been described previously (5). Expression vector pGEX-3X, which allows in-frame ligation of DNA to yield glutathione-S-transferase fusion proteins, was purchased from Pharmacia LKB, Freiburg, Federal Republic of Germany. Expression of the fusion protein is under the control of the isopropyl-β-D-thiogalactopyranoside-inducible p_{tac} promoter. Furthermore, the *lacI*^{q} repressor is located on vector pGEX (28). All plasmid-containing *E. coli* strains were grown in LB broth supplemented with ampicillin (100 μg/ml).

Cloning of the gene coding for hsp60 of *P. aeruginosa*. *P. aeruginosa* chromosomal DNA was extracted by ultracentrifugation as described elsewhere (32). All restriction enzymes (Pharmacia LKB) and T4 DNA ligase (GIBCO BRL)

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TABLE 1. Synthetic oligonucleotides used for epitope mapping

Oligonucleotide	Sequence	Positions of encoded amino acids in GroEL
AS 4	5′ GATCGGTGTG CAGGCTGATA TCGAAGCCCG C 3′	337–345
AS 5	5′ AATTGCGGGC TTCGATATCA GCCTGCACAC C 3′	
AS 9	5′ GATCGATGGC GCCGGTGTGC AGGCTGATAT CGAAGCCCGC GTCCTGCAGA TCCGCAAGCA GATCGAGGAA ACCACT 3′	334–357
AS 10	5′ AATTAGTGGT TTCCTCGATC TGCTTGCGGA TCTGCAGGAC GCGGGCTTCG ATATCAGCCT GCACACCGGC GCCATC 3′	
AS 16	5′ GATCGGTGTG CAGGCTGATA TCGAAGCCCG CGTCCTGCAG 3′	337–348
AS 17	5′ AATTCTGCAG GACGCGGGCT TCGATATCAG CCTGCACACC 3′	
AS 39	5′ GATCGATATC GAAGCCCGCG TCCTGCAG 3′	341–348
AS 40	5′ AATTCTGCAG GACGCGGGCT TCGATATC 3′	
AS 41	5′ GATCGCTGAT ATCGAAGCCC GCGTCCTG 3′	340–347
AS 42	5′ AATTCAGGAC GCGGGCTTCG ATATCAGC 3′	
AS 43	5′ GATCAGGGCT GATATCGAAG CCCGCGTC 3′	339–346
AS 44	5′ AATTGACGCG GGCTTCGATA TCAGCCCT 3′	
AS 49	5′ GATCCAGGCT GATATCGAAG CCCGCGTCCT GCAG 3′	339–348
AS 50	5′ AATTCTGCAG GACGCGGGCT TCGATATCAG CCTG 3′	
AS 51	5′ GATCGTGCAG GCTGATATCG AAGCCCGCGT CCTG 3′	338–347
AS 52	5′ AATTCAGGAC GCGGGCTTCG ATATCAGCCT GCAC 3′	
AS 53	5′ GATCGGTGTG CAGGCTGATA TCGAAGCCCG CGTC 3′	337–346
AS 54	5′ AATTGACGCG GGCTTCGATA TCAGCCTGCA CACC 3′	

were used according to the manufacturers' instructions. A *P. aeruginosa* genomic library was constructed by ligating partially *Sau*3A-digested fragments that were 2 to 4 kb long into the *Bam*HI site of plasmid vector pEMBL8. Screening of this library was performed with monoclonal antibody MAb 2528. This antibody was raised by repeatedly immunizing BALB/c mice with complete cells of *P. aeruginosa* DSM 1707 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Federal Republic of Germany) and was shown to be specific for *P. aeruginosa* hsp60. Specificity for *P. aeruginosa* was demonstrated by the fact that the antibody was reactive with hsp60 preparations from 42 *P. aeruginosa* strains but was not reactive with hsp60 preparations from *Pseudomonas fluorescens*, *Pseudomonas cepacia*, *Xanthomonas maltophilia*, several members of the family *Enterobacteriaceae*, *Acinetobacter* species, *Campylobacter* species, *Neisseria* species, *Haemophilus* species, *Staphylococcus aureus*, and *Streptococcus* species (2). Colony blotting was performed as described previously (18). Plasmid DNA was prepared and restriction analysis and subcloning were performed by using standard procedures (24).

Nested deletions and sequencing. Nested deletions (10) were prepared by using a nested deletion kit purchased from Pharmacia LKB. The sequences of suitable clones were determined by the dideoxy chain termination method, using T7 DNA polymerase, universal primer, and a sequencing kit obtained from Pharmacia LKB. The oligonucleotide primers used for sequencing parts that were not covered by nested deletion clones were synthesized with a Gene Assembler Plus Oligonucleotide synthesizer (Pharmacia LKB). Oligonucleotides were deprotected with 33% NH_4OH. Ammonia and protective groups were removed by using prepacked NAP10 columns (Pharmacia LKB), and oligonucleotides were used directly without further purification steps. Sequencing information was analyzed by using PC/GENE, a software package obtained from Intelligenetics, Inc., Mountain View, Calif.

Epitope mapping using synthetic oligonucleotides. Complementary oligonucleotides with *Bam*HI- and *Eco*RI-compatible ends for appropriate in-frame cloning were synthesized, deprotected, and purified as described above (Table 1). In order to get double-stranded DNA, equal amounts of complementary oligonucleotides were annealed with each other at 37°C for 30 min. pGEX-3X was digested with *Bam*HI and *Eco*RI. Restriction enzymes and the fragments, which had been excised from the cloning site, were removed by phenol extraction and ethanol precipitation. Annealed oligonucleotides were ligated into prepared pGEX-3X at a ratio of insertions to vectors of 10:1. After transformation into strain DH5α, expression of fusion proteins was induced by applying nitrocellulose filters that had been soaked in a 10 mM isopropyl-β-D-thiogalactopyranoside solution to the surfaces of agar plates containing overnight-grown colonies for 2 h. Testing by colony blotting was done as described above.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot procedures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 7.5 and 12.5% gradient polyacrylamide gels by using the discontinuous buffer system of Laemmli (16). All cells were grown to

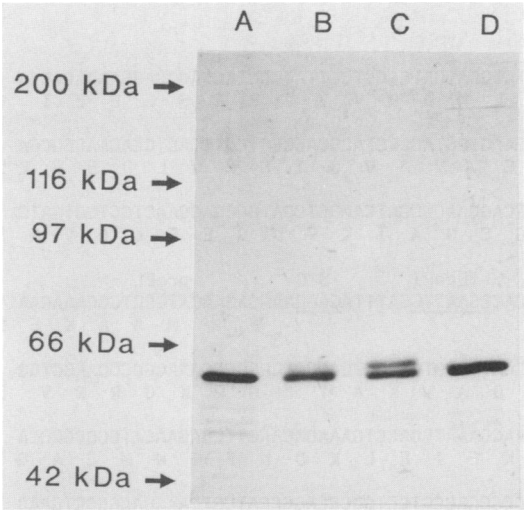

FIG. 1. Immunoblot analysis of the *P. aeruginosa* 60-kDa heat shock protein (hsp60). MAb 2528 was used to identify *P. aeruginosa* hsp60 following transfer of proteins from a 7.5% sodium dodecyl sulfate–polyacrylamide gel to nitrocellulose. Lane A, *P. aeruginosa* ATCC 27853 whole-cell lysate; lane B, *E. coli* DH5α(pAS-1) whole-cell lysate; lane C, *E. coli* DH5α(pAS-2) whole-cell lysate; lane D, *E. coli* BW313(pAS-2) whole-cell lysate. Molecular masses are indicated on the left.

an optical density at 550 nm of 1.2. The cell pellet from a 1-ml culture was resuspended in 100 ml of sample solution (20% glycerol, 3% sodium dodecyl sulfate, 3% 2-mercaptoethanol, 1% bromophenol blue) and then denatured by heating at 100°C for 5 min. Silver staining was performed as described by Switzer et al. (33). Western blotting onto nitrocellulose was performed as described by Towbin and Gordon (34), and blots were immunostained with MAb 2528 and alkaline phosphatase-labeled goat anti-mouse immunoglobulin (Dianova, Hamburg, Federal Republic of Germany). Antibodies were detected by adding substrate containing 0.05 mg of 5-bromo-4-chloro-3-indolylphosphate per ml, 0.1 mg of Nitro Blue Tetrazolium (in 100 mM Tris hydrochloride, pH 9.6) per ml, and 4 mM $MgCl_2$.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited in the GenBank/EMBL data bank under accession number M63957.

RESULTS

Cloning of the gene coding for hsp60 of *P. aeruginosa*. The genomic library of *P. aeruginosa* constructed in *E. coli* DH5α by using pEMBL8 was screened by colony blotting for clones that were reactive with *P. aeruginosa* hsp60-specific MAb 2528. Of approximately 9,000 colonies screened, 2 were found to be positive. These colonies were picked, and one clone was chosen for further study. This clone, designated pAS-1, was found in Western blots to express large amounts of a protein which was detected by MAb 2528 and had the same molecular weight as hsp60 of *P. aeruginosa* (Fig. 1). A restriction analysis of the plasmid DNA of pAS-1 showed that we had cloned a fragment that was approximately 8 kb long. As the library was constructed by ligating 2- to 4-kb *Sau*3A fragments in pEMBL8, pAS-1 presumably contained at least two of these fragments. Digestion of pAS-1 with *Eco*RI yielded three fragments, which were 6, 4, and 2 kb long. As the 6-kb *Eco*RI fragment was shown by religation to contain the vector, only the 4- and 2-kb *Eco*RI fragments were subcloned into pEMBL8. We found that the clone containing the 2-kb *Eco*RI fragment expressed a protein which was recognized by MAb 2528 and had the same molecular weight as the protein expressed by pAS-1 and the hsp60 of *P. aeruginosa* (Fig. 1). This clone was designated pAS-2.

Sequence analysis. We determined the sequence of the *Eco*RI fragment in pAS-2 by sequencing overlapping nested deletion clones generated from pAS-2. Parts not covered by the nested deletion were sequenced with synthetic oligonucleotide primers. Oligonucleotide primers were also used for sequencing the gene coding for hsp10 of *P. aeruginosa* in pAS-1. The sequence analysis revealed that there were two open reading frames that were separated by 50 bases (Fig. 2). These open reading frames could encode proteins that were 97 and 547 amino acids long and had calculated molecular masses of 10 and 57 kDa, respectively. In analogy to the *groE* operon of *E. coli*, we designated the gene that coded for the 10-kDa protein *groES*, the gene that coded for the 57-kDa protein *groEL*, and the proteins that were encoded by these genes GroES and GroEL, respectively. Initiation codons are preceded by regions which could represent ribosomal binding sites (7). *groEL* is followed by a palindromic sequence that resembles a rho-independent transcriptional terminator. When we tested pAS-2, MAb 2528 detected double bands (Fig. 1). A sequence analysis revealed that in contrast to pAS-1, which harbored the complete *groE* operon, pAS-2 contained only *groEL*. In pAS-2 the ATG start codon of *groEL* was found 20 nucleotides downstream from the *Eco*RI cloning site (Fig. 2). About 17 nucleotides upstream from the *Eco*RI site there was another ATG start codon present in pEMBL8 (data not shown). This start codon was preceded by a region that may serve as a ribosomal binding site and is in frame with *groEL*. Therefore, expression of an additional somewhat larger heat shock protein might start at this second start codon. Although there was a TAG stop codon located between the *Eco*RI site and the *groEL* start codon, this should have been translated to glutamine in *E. coli* DH5α, as this strain contains a *supE* gene. In order to prove this hypothesis, we transformed pAS-2 into nonsuppressing *E. coli* strain BW313. In this strain expression of the larger band ceased, and only one band with the same molecular weight as that of the hsp60 expressed by pAS-1 was detected by MAb 2528 (Fig. 1).

Our analysis of the derived amino acid sequence of GroEL revealed a typical chaperonin signature at positions 405 to 416 (1) (Fig. 2). Homology searches in which we used the derived amino acid sequences encoded by *groES* and *groEL* disclosed striking identity to the group containing hsp10 and hsp60 proteins. As Fig. 3 shows, alignments of the derived amino acid sequence of GroEL of *P. aeruginosa* with the amino acid sequences of previously described hsp60 proteins revealed 78% identity with the GroEL protein of *E. coli* (9), 75% identity with the htpB proteins of *L. pneumophila* (11) and *Coxiella burnetii* (35), 58% identity with the mycobacterial 65-kDa antigens (17, 26), and 61% identity with the hypB protein of *Chlamydia psittaci* (19). A nucleotide sequence comparison of the complete pseudomonal *groE* operon revealed levels of identity of 73.7% with *groE* of *E. coli*, 60.7% with the *groE* homolog of *L. pneumophila*, 64.6% with the *Coxiella burnetti* sequence, 66.5% with the *Mycobacterium tuberculosis* sequence, and 60.1% with the *Chlamydia psittaci* sequence (data not shown).

Epitope mapping. To map the epitope recognized by MAb

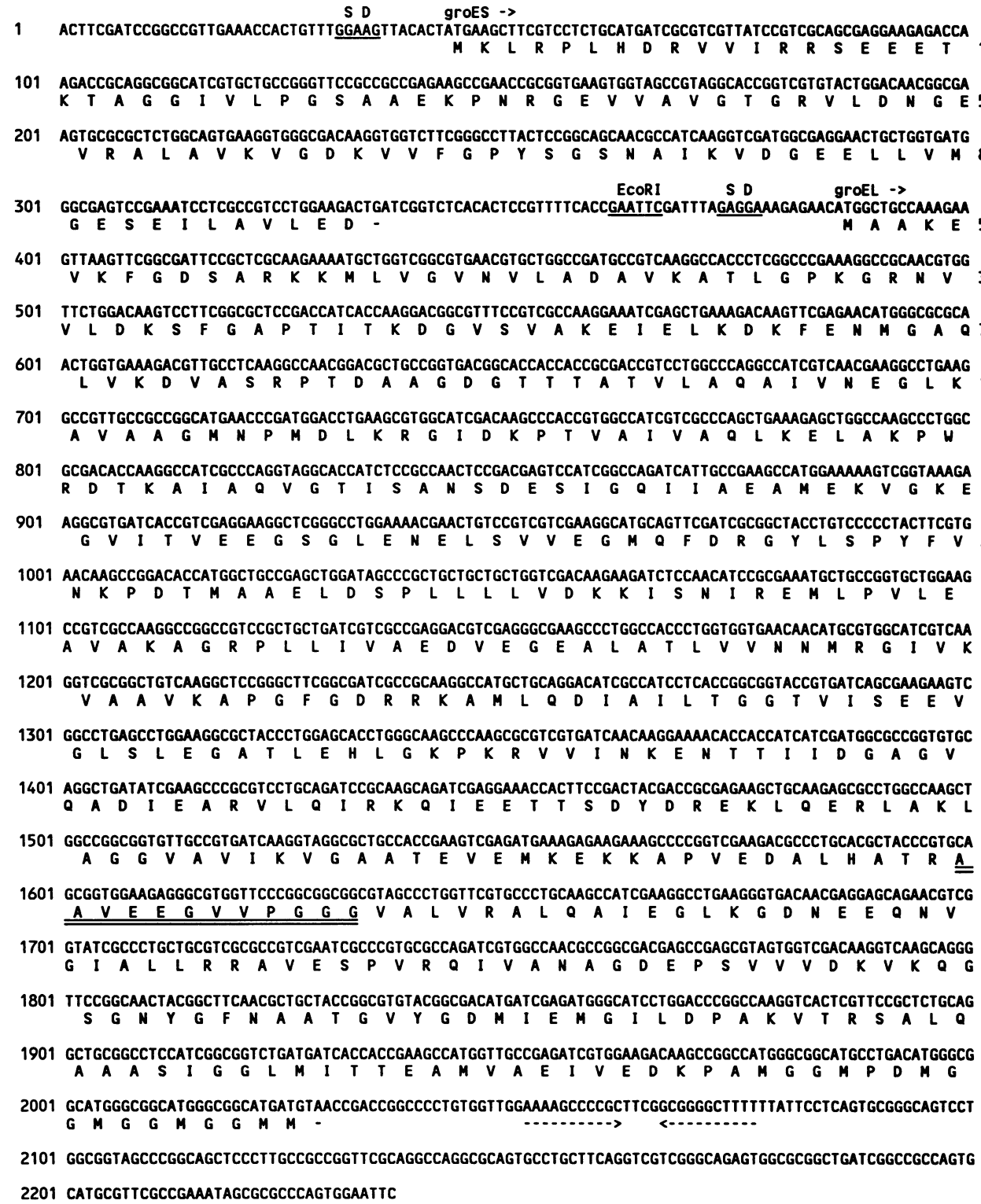

FIG. 2. Nucleotide sequence of the *P. aeruginosa groE* operon. The amino acid sequences translated from the *groES* and *groEL* genes are indicated below the nucleotide sequence. Putative Shine-Dalgarno sites are underlined and labeled SD. The *Eco*RI site used for subcloning of *groEL* in pAS-2 is underlined in the intergenic region. A typical chaperonin signature at positions 405 to 416 of GroEL is indicated by double underlining (1). The 11-bp dyad symmetry of the proposed transcription termination site is indicated by arrows. The numbers on the left are the nucleotide sequence numbers, and the numbers on the right are the amino acid positions in each protein.

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Chl. ps.    MAAKNIKYNEDARKKIHKGVKTLAEAVKVTLGPKGRHVVIDKSFGSPQVTKDGVTVAKEIELEDKHENMGAQMVKEVASKTADKAGDGTTTATVLAEAIY 100
Cox. bur.   MAAKVLKFSHEVLHAMSRGVEVLANAVKVTLGPKGRNVVLDKSFGAPTITKDGVSVAKEIELEDKFENMGAQMVKEVASRTSDDAGDGTTTATVLAQAIL 100
Leg. pn.    MA-KELRFGDDARLQMLAGVNALADAVQVTMGPRGRNVVLEKSYGAPTVTKDGVSVAKEIEFEHRFMNMGAQMVKEVASKTSDTAGDGTTTATVLARSIL  99
E. coli     MAAKDVKFGNDARVKMLRGVNVLADAVKVTLGPKGRNVVLDKSFGAPTITKDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTTTATVLAQAII 100
My. lep.    MA-KTIAYDEEARRGLERGLNSLADAVKVTLGPKGRNVVREKKWGAPTITNDGVSIAKEIELEDPYEKIGAELVKEVAKKTDDVAGDGTTTATVLAQALV  99
My. tub.    MA-KTIAYDEEARRGLERGLNALADAVKVTLGPKGRNVVLEKKWGAPTITNDGVSIAKEIELEDPYEKIGAELVKEVAKKTDDVAGDGTTTATVLAQALV  99
Ps. aerug.  MAAKEVKFGDSARKKMLVGVNVLADAVKATLGPKGRNVVLDKSFGAPTITKDGVSVAKEIELKDKFENMGAQLVKDVASRPTDAAGDGTTTATVLAQAIV 100
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Chl. ps.    SEGLRNVTAGANPMDLKRGIDKAVKVVVDEIKKISKPVQHHKEIAQVATISANNDAEIGNLIAEAMEKVGKNGSITVEEAKGFETVLDVVEGMNFNRGYL 200
Cox. bur.   VEGIKAVIAGMNPMDLKRGIDKAVTAAVAELKKISKPCKDQKAIAQVGTISANSDKSIGDIIAEAMEKVGKEGVITVEDGSGLENALEVVEGMQFDRGYL 200
Leg. pn.    VEGHKAVAAGMNPMDLKRGIDKAVLAVTKKLQAMSKPCKDSKAIAQVGTISANSDEAIGAIIAEAMEKVGKEGVITVEDGNGLENELSVVEGMQFDRGYI 199
E. coli     TEGLKAVAAGMNPMDLKRGIDKAVTAAVEELKALSVPCSDSKAIAQVGTISANSDETVGKLIAEAMDKVGKEGVITVEDGTGLQDELDVVEGMQFDRGYL 200
My. lep.    KEGLRNVAAGANPLGLKRGIEKAVDKVTETLLKDAKEVETKEQIAATAAISA-GDQSIGDLIAEAMDKVGNEGVITVEESNTFGLQLELTEGMRFDKGYI 198
My. tub.    REGLRNVAAGANPLGLKRGIEKAVEKVTETLLKGAKEVETKEQIAATAAISA-GDQSIGDLIAEAMDKVGNEGVITVEESNTFGLQLELTEGMRFDKGYI 198
Ps. aerug.  NEGLKAVAAGMNPMDLKRGIDKPTVAIVAQLKELAKPWRDTKAIAQVGTISANSDESIGQIIAEAMEKVGKEGVITVEEGSGLENELSVVEGMQFDRGYL 200
             **   * ** **  ***** *                     **    ***  *   *  ***** ***  * ****        *   *** *  **

Chl. ps.    SSYFSTNPETQECVLEEALVLIYDKKISGIKDFLPVLQQVAESGRPLLIIAEDIEGEALATLVVNRLRAGFRVCAVKAPGFGDRRKAMLEDIAILTGGQL 300
Cox. bur.   SPYFINNQQNMSAELENPFILLVDKKISNIRELIPLLENVAKSGRPLLVIAEDIEGEALATLVVNNIRGVVKVAAVKAPGFGDRRKAMLQDIAVLTGGKV 300
Leg. pn.    SPYFINNQQNMSCELEHPFILLVDKKVSSIREMLSVLEGVAKSGRPLLIIAEDVEGEALATLVVNNMRGIVKVCAVKAPGFGDRRKAILQDIAILTKGQV 299
E. coli     SPYFINKPETGAVELESPFILLADKKISNIREMLPVLEAVAKAGKPLLIIAEDVEGEALATAVVNTIRGIVKVAAVKAPGFGDRRKAMLQDIATLTGGTV 300
My. lep.    SGYFVTDAERQEAVLEEPYILLVSSKVSTVKDLLPLLEKVIQAGKSLLIIAEDVEGEALSTLVVNKIRGTFKSVAVKAPGFGDRRKAMLQDMAILTGAQV 298
My. tub.    SGYFVTDPERQEAVLEDPYILLVSSKVSTVKDLLPLLEKVIGAGKPLLIIAEDVEGEALSTLVVNKIRGTFKSVAVKAPGFGDRRKAMLQDMAILTGGQV 298
Ps. aerug.  SPYFVNKPDTMAAELDSPLLLLVDKKISNIREMLPVLEAVAKAGRPLLIVAEDVEGEALATLVVNNMRGIVKVAAVKAPGFGDRRKAMLQDIAILTGGTV 300
            * **          *     *    * *        *  *   *  **  *** ***** * ***  *      ************* * * * **

Chl. ps.    ISEELGMKLENTTLAMLGKAKKVIVSKEDTTIVEGLGSKEDIESRCESIKKQIEDSTSDYDKEKLQERLAKLSGGVAVIRVGAATEIEMKEKKDRVDDAQ 400
Cox. bur.   ISEEVGLSLEAASLDDLGSAKRVVVTKDDTTIIDGSGDAGDIKNRVEQIRKEIENSSSDYDKEKLQERLAKLAGGVAVIKVGAATEVEMKEKKARVEDAL 400
Leg. pn.    ISEEIGKSLEGATLEDLGSAKRIVVTKENTTIIDGEGKATEINARITQIRAQMEETTSDYDREKLQERVAKLAGGVAVIKVGAATEVEMKEKKAAVEDAL 399
E. coli     ISEEIGMELEKATLEDLGQAKRVVINKDTTTIIDGVGEEAAIQGRVAQIRQQIEEATSDYDREKLQERVAKLAGGVAVIKVGAATEVEMKEKKARVEDAL 400
My. lep.    ISEEVGLTLENTDLSLLGKARKVVMTKDETTIVEGAGDTDAIAGRVAQIRTEIENSDSDYDREKLQERLAKLAGGVAVIKAGAATEVELKERKHRIEDAV 398
My. tub.    ISEEVGLTLENADLSLLGKARKVVVTKDETTIVEGAGDTDAIAGRVAQIRQEIENSDSDYDREKLQERLAKLAGGVAVIKAGAATEVELKERKHRIEDAV 398
Ps. aerug.  ISEEVGLSLEGATLEHLGKPKRVVINKENTTIIDGAGVQADIEARVLQIRKQIEETTSDYDREKLQERLAKLAGGVAVIKVGAATEVEMKEKKAPVEDAL 400
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Chl. ps.    HATLAAVEEGILPGGGTALVRCIPTLEAFIPILTNEDEQIGARIVLKALSAPLKQIAANAGKEGAIICQQVLSRSSSE-GYDALRDAYTDMIEAGILDPT 499
Cox. bur.   HATRAAVEEGVVPGGGVALIRVLKSLDSVEV--ENEDQRVGVEIARRAMAYPLSQIVKNTGVQAAVVADKVLNHKDVNYGYNAATGEYGDMIEMGILDPT 498
Leg. pn.    HATRAAVEEGIVAGGGVALIRAQKALDSLKG--DNDDQNMGINILRRAIESPMRQIVTNAGYEASVVVNKVAEHKD-NYGFNAATGEYGDMVEMGILDPT 496
E. coli     HATRAAVEEGVVAGGGVALIRVASKLADLRG--QNEDQNVGIKVALRAMEAPLRQIVLNCGEEPSVVANTV-KGGDGNYGYNAATEEYGNMIDMGILDPT 497
My. lep.    RNAKAAVEEGIVAGGGVTLLQAAPALDKLK--LRPDEAT-GANIVKVALEAPLKQIAFNSGMEPGVVAEKVRNLSVGH-GLNAATGEYEDLLKAGVADPV 494
My. tub.    RNAKAAVEEGIVAGGGVTLLQAAPTLDELK--LEGDEAT-GANIVKVALEAPLKQIAFNSGLEPGVVAEKVRNLPAGH-GLNAQTGVYEDLLAAGVADPV 494
Ps. aerug.  HATRAAVEEGVVPGGGVALVRALQAIEGLKG--DNEEQNVGIALLRRAVESPVRQIVANAGDEPSVVVDKV-KQGSGNYGFNAATGVYGDMIEMGILDPA 497
                ******   ***  *                     *      *   *  **  * *         *        *  *    *      *  **

Chl. ps.    KVTRCALESAASVAGLLLTTEALIADIPEEKSSSAPA---MPG-------AGMDY   544
Cox. bur.   KVTRTALQNAASIAGLMITTECMVTEAPKKKEESMPGGGDMGGMGGMGGMGGMM-   552
Leg. pn.    KVTRMALQNAASVASLMLTTECMVADLPKKEEGV--GAGDMGGMGGMGGMGGMMH   549
E. coli     KVTRSALQYAASVAGLMITTECMVTDLPKNDAADL---GAAGGMGGMGGMGGMM-   548
My. lep.    KVTRSALQNAASIAGLFLTTEAVVADKPEKTAAPASDPT--------GGMGGMDF   541
My. tub.    KVTRSALQNAASIAGLFLTTEAVVADKPEKEKASVPG-G--------GDMGGMDF   540
Ps. aerug.  KVTRSALQAAASIGGLMITTEAMVAEIVEDKPA-M---GGMPDMGGMGGMGGMM-   547
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FIG. 3. Comparison of the deduced amino acid sequences of the 60-kDa heat shock proteins of several bacterial species. The deduced amino acid sequences for hypB of *Chlamydia psittacii* (Chl. ps.) (19), htpB of *Coxiella burnetii* (Cox. bur.) (35) and *Legionella pneumophila* (Leg. pn.) (11), GroEL of *E. coli* (E. coli) (9), 65-kDa proteins of *Mycobacterium leprae* (My. lep.) (17) and *M. tuberculosis* (My. tub.), and GroEL of *P. aeruginosa* (Ps. aerug.) are shown. The asterisks indicate the presence of identical amino acids.

2528 on hsp60 of *P. aeruginosa*, we used the nested deletion clones generated for sequencing. As deletions had been made from the part of *groEL* which codes for the carboxy terminus of the GroEL protein, these clones expressed a series of proteins which represented GroEL lacking a successively larger part of its carboxy terminus. By testing the proteins expressed by these clones in Western blots and sequencing the exact end of the insertion of each clone, the last clone still detected by MAb 2528 and the first negative clone in the series defined the borders for our search (Fig. 4).

In order to look for single amino acid residues or groups of amino acid residues that were specific for *P. aeruginosa*, we aligned the region of *P. aeruginosa* GroEL delimited by the ends of positive nested deletion clone X16 and negative clone X13 with the same region of previously described hsp60 proteins. Several *P. aeruginosa*-specific amino acid residues were found between positions 337 and 351 (Fig. 5). Oligonucleotides with *Bam*HI- and *Eco*RI-compatible ends that coded for short peptides were synthesized. The sequences of these peptides were taken from the region defined by our previous findings and so may have contained the *P. aeruginosa*-specific epitope recognized by MAb 2528. These oligonucleotides were ligated into the *Bam*HI-*Eco*RI site of pGEX-3X and expressed as glutathione-S-transferase fusion proteins in *E. coli* DH5α. Transformants were tested by colony blotting, using MAb 2528. Positive clones were picked, plasmid DNA was prepared, and the sequence of the insertion was determined. Reactivity with MAb 2528 was confirmed by Western blotting. If there were no positive colonies, clones were picked randomly until at least one was

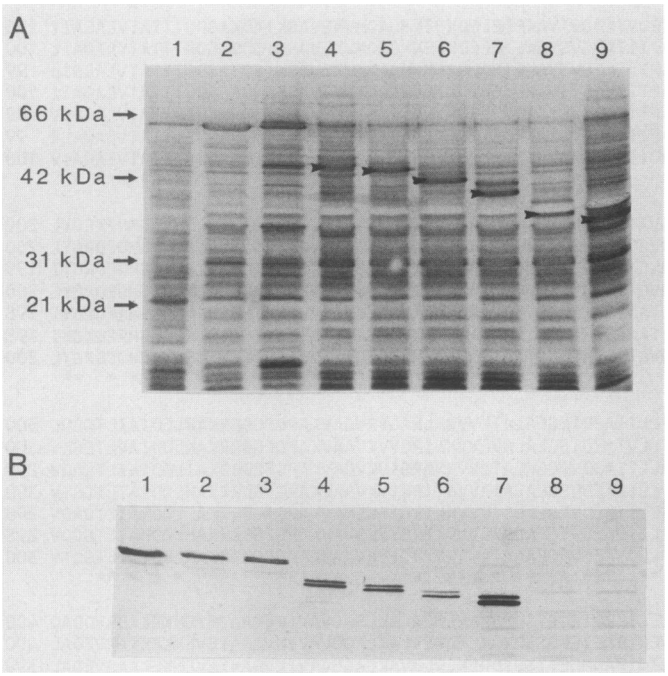

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis of a series of nested deletion clones derived from pAS-2. (A) Silver-stained 12.5% sodium dodecyl sulfate–polyacrylamide gel. The bands corresponding to truncated hsp60 proteins expressed by pAS-2-derived nested deletion clones are indicated by arrowheads. (B) Western blot immunostained with *P. aeruginosa* hsp60-specific MAb 2528. Lane 1, *P. aeruginosa* ATCC 27853 whole-cell lysate; lane 2, *E. coli* DH5α(pAS-1) whole-cell lysate; lane 3, *E. coli* DH5α(pAS-2) whole-cell lysate; lanes 4 through 9, whole-cell lysates of *E. coli* DH5α(pAS-2)-derived nested deletion clones. Clone X16 (lane 7) was the last in the series which was still recognized by MAb 2528. Clone X13 (lane 8) and the following clones exhibited no reactivity.

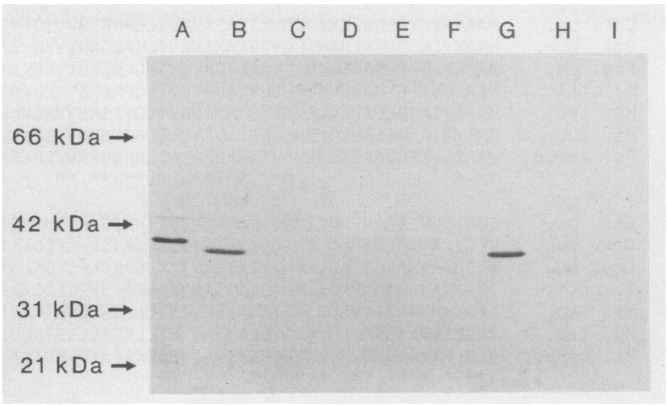

FIG. 6. Western blot of whole-cell lysates of *E. coli* DH5α harboring modified pGEX-3X plasmids that express short peptides as parts of glutathione-S-transferase fusion proteins for epitope mapping. The blot was immunostained with *P. aeruginosa* hsp60-specific MAb 2528. The clones were constructed by using the following oligonucleotides: lane A, AS 9 and AS 10; lane B, AS 16 and AS 17; lane C, AS 4 and AS 5; lane D, AS 39 and AS 40; lane E, AS 41 and AS 42; lane F, AS 43 and AS 44; lane G, AS 49 and AS 50; lane H, AS 51 and AS 52; lane I, AS 53 and AS 54. Expression of fusion protein was controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining (data not shown). All of the clones tested in this Western blot experiment expressed equal amounts of fusion proteins.

shown to contain the correct insertion while being negative on Western blots.

By using the strategy described above we were able to show that a region of 24 amino acid residues (positions 334 to 357 on GroEL of *P. aeruginosa*) contained the species-specific epitope (Fig. 5 and 6). A second clone that was constructed with oligonucleotides AS 16 and AS 17 and expressed 12 amino acid residues corresponding to positions 337 to 348 of GroEL also exhibited reactivity with MAb 2528. However, a clone that expressed 9 of these 12 residues (constructed with oligonucleotides AS 4 and AS 5) was negative. As the present view is that sequential epitopes are from five to eight residues long (6), we then created three additional clones by using oligonucleotides AS 39 and AS 40,

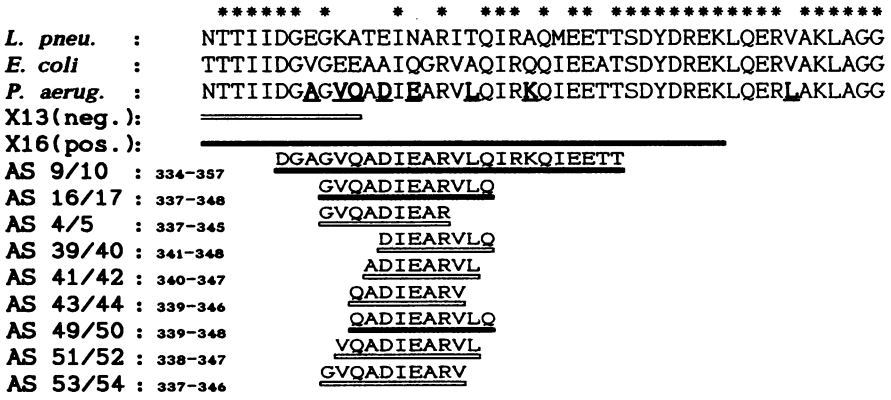

FIG. 5. Schematic representation of the strategy used for mapping the epitope recognized by MAb 2528. The deduced amino acid sequence of *P. aeruginosa* (P. aerug.) GroEL positions 329 to 375 and the corresponding sequences of *L. pneumophila* (L. pneu.) htpB and *E. coli* (E. coli) GroEL are shown. The asterisks indicate identity, and *P. aeruginosa*-specific amino acid residues are underlined. The ends of pAS-2-derived nested deletion clones X13 and X16 and the locations of the short peptide sequences expressed as glutathione-S-transferase fusion proteins are indicated by bars. The solid bars indicate clones recognized by MAb 2528; nonreactive clones are indicated by open bars. The exact positions of the peptide sequences within GroEL of *P. aeruginosa* are indicated by the numbers to the left of the bars.

oligonucleotides AS 41 and AS 42, and oligonucleotides AS 43 and AS 44. These clones expressed fusion proteins containing the remaining possible octapeptides. None of these candidates proved to be positive. Therefore, we next constructed a series of clones that expressed fusion proteins by using oligonucleotides AS 49 and AS 50, oligonucleotides AS 51 and AS 52, and oligonucleotides AS 53 and AS 54; these clones produced four decapeptides. This time we found that one clone constructed with AS 49 and AS 50, which expressed the decapeptide QADIEARVLQ, corresponding to *P. aeruginosa* GroEL positions 339 to 348, reacted with MAb 2528 (Fig. 5 and 6).

DISCUSSION

In this study we cloned and sequenced the genes that code for the 10- and 60-kDa heat shock proteins of *P. aeruginosa*. Homologs of hsp60, which are found in prokaryotes, chloroplasts, and mitochondria, seem to play an essential and ubiquitous role in the posttranslational assembly of proteins (9). This might explain the high degree of amino acid sequence similarity among chaperonins, which is observed even between very distant species. The hsp60 of *P. aeruginosa* is yet another example of this striking similarity. Alignment with several previously known amino acid sequences from different species showed that the chaperonin of *P. aeruginosa* fits in very well, exhibiting not only the typical chaperonin signature but also overall levels of identity ranging from 58 to 78% with previously described prokaryotic sequences. Therefore, the complete sequence described in this paper explains the previously observed broad cross-reactivity of the common antigen of *P. aeruginosa* on the molecular level (12).

Except for the mycobacterial 65-kDa antigens, all known prokaryotic hsp60 genes have been found to be part of an operon, which consists of the genes for hsp10 and hsp60. The *groES* and *groEL* genes of *E. coli* have been shown to be cotranscribed. These proteins form a complex with each other in the presence of Mg-ATP (9) and are both required for the correct assembly of proteins (8). The *groES* and *groEL* genes of *P. aeruginosa* described in this paper have the same arrangement as all of the other prokaryotic hsp10-hsp60 operons; *groES* is found upstream from *groEL*.

Although the most striking features of hsp60 molecules are their immunudominance and broad cross-reactivity, it has been shown that these proteins also contain genus- and species-specific epitopes (31). The monoclonal antibody, MAb 2528, used in this study was shown in extensive tests to recognize a species-specific epitope on the hsp60 of *P. aeruginosa* (2). As binding of MAb 2528 occurred under conditions which should destroy the natural conformation of the protein, it seemed likely that the antibody recognizes a continuous epitope and not a conformational epitope. Therefore, we combined two strategies for mapping, testing of nested deletion clones to define the gross range for a further search and construction of pGEX-3X clones that express fusion proteins which contain peptides that may resemble the epitope. By using this method we showed that amino acid residues 339 to 348 (QADIEARVLQ) are responsible for binding of MAb 2528. Although with the series of fusion proteins tested in this study we were not able to decide whether the first residue of this decapeptide is essential for binding, we found that the three remaining possible octapeptide epitopes within this range were not sufficient for antibody binding in our test system. This finding is an exception to the view formulated by Geysen et al. that sequential epitopes are from five to eight residues long (6). Thus, our finding indicates that nona- and decapeptides should be considered possible candidates when epitopes are mapped. The present practice of using only octapeptides for epitope scanning might lead to omission of some epitopes.

The hsp60 family comprises highly immunodominant molecules which have been found to represent the major stress protein antigen recognized by antibodies in bacterial infections (37). In a study of the 58-kDa common antigen of *L. pneumophila*, this antigen was found to be the only antigen which was recognized by all sera from patients with culture-confirmed legionellosis (25). All of these findings suggest that species-specific hsp60 epitopes may be prime candidates for the development of serological tests. A recent study of the hsp60 of *Chlamydia trachomatis* showed that the antibody reactivity present in human sera against hsp60 is predominantly directed toward the carboxy-terminal one-half of the *Chlamydia trachomatis* hsp60 molecule (3). The species-specific epitope recognized by MAb 2528 is located between two highly conserved regions in the carboxy-terminal one-half of the hsp60 of *P. aeruginosa* and might therefore be recognized by antibodies produced by patients with pseudomonal infections. The detection of the onset of *P. aeruginosa* infections with serological tests has been demonstrated in patients suffering from cystic fibrosis (21). Therefore, further studies should show whether the epitope defined in this study might prove to be useful for the development of *P. aeruginosa*-specific serological tests.

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