BACTERIAL CHARACTERISATION

Cloning, sequencing, characterisation and implications for vaccine design of the novel dihydrolipoyl acetyltransferase of *Neisseria meningitidis*

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A λ Zap-II expression library of *Neisseria meningitidis* was screened with a rabbit polyclonal antiserum (R-70) raised against *c.* 70-kDa proteins purified from outer membrane vesicles by elution from preparative SDS-polyacrylamide gels. Selected clones were isolated, further purified, and their recombinant pBluescript SKII plasmids were excised. The cloned DNA insert was sequenced from positive clones and analysed. Four open reading frames (ORFs) were identified, three of which showed a high degree of homology with the pyruvate dehydrogenase (E1p), dihydrolipoyl acetyltransferase (E2p) and dihydrolipoyl dehydrogenase (E3) components of the pyruvate dehydrogenase complex (PDHC) of a number of prokaryotic and eukaryotic species. Sequence analysis indicated that the meningococcal E2p (Men-E2p) contains two N-terminal lipoyl domains, an E1/E3 binding domain and a catalytic domain. The domains are separated by hinge regions rich in alanine, proline and charged residues. Another lipoyl domain with high sequence similarity to the Men-E2p lipoyl domain was found at the N-terminal of the E3 component. A further ORF, coding for a 16.5-kDa protein, was found between the ORFs encoding the E2p and E3 components. The identity and functional characteristics of the expressed and purified heterologous Men-E2p were confirmed as dihydrolipoyl acetyltransferase by immunological and biochemical assays. N-terminal amino-acid analysis confirmed the sequence of the DNA-derived mature protein. Purified Men-E2p reacted with monospecific antisera raised against the whole E2p molecule and against the lipoyl domain of the *Azotobacter vinelandii* E2p. Conversely, rabbit antiserum raised against Men-E2p reacted with protein extracts of *A. vinelandii*, *Escherichia coli* and *N. gonorrhoeae* and with the lipoyl and catalytic domains of E2p obtained by limited proteolysis. In contrast, the original R-70 antiserum reacted almost exclusively with the lipoyl domain, indicating the strong immunogenicity of this domain. Antibodies to Men-E2p were detected in patients and animals (rabbits and mice) infected with homologous or heterologous meningococci or other neisserial species. These results have important implications for the understanding of PDHC and the design of future outer membrane vesicle-based vaccines.

Introduction

Meningococci produce a relatively complex outer membrane that contains a large number of proteins

(outer-membrane proteins, OMPs). Many of these proteins are expressed constitutively, but others are environmentally regulated. Recently, there has been a major focus on OMPs for vaccine production, particularly against group B meningococci, and a number of relatively crude OMP mixtures were tested in clinical trials with various degrees of reported efficacy [1]. Following large scale placebo-controlled, randomised double-blind trials, only the vaccines produced in

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Norway and Cuba showed significant protective efficacy. The Norwegian vaccine consisted of OMPs from the Norwegian epidemic strain of *Neisseria meningitidis*, B:15:P1.7.16 and was given to children aged 14–16 years. It produced a point estimate of protective efficacy of 57% after a 30-month follow-up and was, therefore, considered insufficiently effective for general use [2]. The Cuban vaccine consisted of group C capsular polysaccharide mixed with OMPs from a Cuban epidemic strain, B:4:P1.15. When given to children aged 10–16 years, it offered an estimated point efficacy of 83% after 16 months of follow-up [3] and, as a result, the vaccine is now incorporated into the routine childhood vaccination programme in Cuba. Although the Cuban trial did not directly address efficacy in children <10 years old, follow-up studies of the mass vaccination have suggested that the overall protective efficacy based on vaccine coverage and incidence of disease in children under 6 years old is *c.* 93% [3]. However, when the Cuban vaccine was tested in a case-control study in Brazil, protective efficacy was reported to vary with age. The vaccine was effective in children aged 4 years and older, but not in younger children [4].

Although these OMP vaccine preparations are enriched for major class antigens such as serotype (class 2/3) and serosubtype (class 1) OMPs, they also contain significant amounts of other uncharacterised proteins. This makes the search for component(s) relevant to protection in man, and standardisation of preparations for future use, extremely complicated. Moreover, most of the OMP vaccines used consist of outer membrane vesicle (OMV) preparations [2–4]. Such OMVs are likely to include transmembrane, inner membrane or even cytoplasmic components. It is not known which of these components contribute most to antibody-mediated or cell-mediated immune responses and long-term protection from disease. It is possible that these OMVs contain a mixture of antigens, with varying regulating effects on the immune system. Furthermore, there are numerous cell components which are structurally, functionally or immunologically conserved across prokaryotic and eukaryotic cells and which may have been included, unwittingly, in the OMV vaccine preparations. Inclusion of such components in vaccines will carry the risk of breaking immune tolerance with concomitant risk of inducing autoimmune disease. Therefore, more information is desirable on the individual meningococcal OMV antigens in terms of structure and function to facilitate development of an ideal meningococcal vaccine.

OMVs consisting of a wide range of outer-membrane and other proteins were prepared in an earlier study. Purified proteins of *c.* 70-kDa were prepared from these OMVs by elution from preparative SDS-polyacrylamide gels and the antigens were used to raise rabbit polyclonal antiserum (R-70) [5]. Immuno-

blot experiments showed that this antiserum contained antibodies against several co-migrating meningococcal proteins, including the iron-regulated FrpB and non iron-regulated proteins [5, 6]. The exact identity of these different co-migrating proteins has been unclear.

We, and others, have previously characterised FrpB and demonstrated that it is a partially surface-exposed OMP [5–7]. More recently, Pettersson *et al.* [8] cloned and sequenced FrpB and showed that it is related to the family of TonB-dependent proteins. However, its functional attributes remain largely unclear. With regard to the non-iron-regulated 70-kDa protein(s), very little is known. Aoun *et al.* [9] showed that murine sera raised against live meningococci were bactericidal to gonococci and reacted preferentially (in immunoblots) with a highly conserved and constitutively expressed 70-kDa protein. It is not clear whether this immunoreactive 70-kDa protein and the one(s) previously purified in our laboratories [6, 10] are the same.

Therefore, in this study the R-70 antiserum was used to screen a DNA expression library in an attempt to clone and further identify those OMV components that were recognised by this polyclonal rabbit antiserum. The identification, sequence analysis and functional determination of one of these reactive components, the dihydrolipoyl acetyltransferase component of the pyruvate dehydrogenase complex (PDHC) is reported here. The detection of a gene cluster which consists of all the necessary components of the PDHC as well as a unique reading frame which has not been detected in pathogenic organisms is also reported.

Materials and methods

Bacterial strains, vectors and total protein extraction

N. meningitidis strains SD (B:15:P1.16), AS (A:4:P1.7) and EB (29E) were grown in Mueller Hinton broth (MHB) overnight at 37°C in air with CO₂ 5%. Iron-restriction, where required, was achieved by the addition of 25 µM desferrioxamine (Ciba-Giegy, Horsham). *Escherichia coli* strain XL1 Blue was obtained from Stratagene (Cambridge). *E. coli* strain TG2, a *recA*[−] version of TG1, i.e., *thi*[−]1, *supE*44, [Res[−] Mod[−] (k)], F (*traD*36 *pro* A⁺B⁺), *laqI*^{qZa}ΔM15 Δ(*lac-pro*), was used for cloning and expression of plasmid-encoded proteins [11]. Total protein extracts from *N. meningitidis* strain SD and *E. coli* XL1-Blue carrying plasmid, before or after isopropyl β-D thiogalactopyranoside (IPTG)-induction, were prepared as described previously [12].

Sera, SDS-PAGE and immunoblots

The rabbit antiserum against purified *c.* 70-kDa OMV proteins of meningococci (R-70) was raised and

characterised previously [10]. Before use, the R-70 antiserum was adsorbed once with immobilised *E. coli* lysate (Pierce, Pierce and Warriner, Chester) as described by the manufacturers. Rabbit antisera to whole cells (live organisms) of meningococcal strains SD, AS, EB and the commensal *N. lactamica* strain were raised in New Zealand White rabbits as described previously [5]. Rabbit antiserum to meningococcal E2p (Men-E2p, see below) was raised by inoculating a New Zealand White rabbit, by subcutaneous injection, with five doses of 100 µg of purified protein (eluted from SDS-polyacrylamide gels) suspended in 500 µl of phosphate-buffered saline (pH 7.2; PBS, Oxoid) and mixed with equal volumes of Freund's complete (first injection) and incomplete (the subsequent fortnightly booster injections) adjuvants. Pre-immune normal rabbit serum was also obtained. Murine polyclonal antisera to live cells of meningococcal strains SD and B16B6 (B:2a:P1.2) and murine monoclonal anti-FrpB (Mab-M70) were raised and characterised previously [6, 13]. Pre-immune normal mouse serum was also obtained. Human convalescent sera were taken from patients 4 weeks after recovery from meningococcal disease (meningitis or septicaemia, or both) caused by different meningococcal strains, including strain SD, JB (B:NT) and GLD (C:2a:P1.10). A human convalescent serum was also obtained from a patient who had recovered from gonococcal pelvic inflammatory disease. Normal human serum was obtained from an individual with no prior history of infection with *N. meningitidis* or *N. gonorrhoeae*.

SDS-PAGE and immunoblots of the rabbit, murine and human antisera against total protein extracts and purified Men-E2p (below) were performed as described previously [14].

Gene library screening, cloning and sequencing

The R-70 antiserum was used to screen a previously constructed gene library of *N. meningitidis* (strain SD) DNA in λZap II [12]. Approximately 50 000 plaques, seeded on *E. coli* strain XL1 Blue, were lifted in duplicates on to nitrocellulose membranes (Schleicher and Schuell, supplied by Anderman, Kingston-upon-Thames) pre-impregnated with 0.5 mM IPTG. As the rabbit R-70 antiserum was raised against denatured meningococcal OMV proteins, one copy of the plaque-life membrane was denatured by heating at 56°C for 30 min in protein-denaturation buffer (containing SDS 1% and 2-mercaptoethanol 0.2%) and the other copy was left untreated. Both membranes were blocked in blocking buffer (bovine serum albumin 3%, Tween 20 0.1% in PBS) for 1 h, and then incubated with the R-70 antiserum (diluted 1 in 1000) for 3 h at room temperature followed by peroxidase conjugated goat anti-rabbit serum as previously described [5, 10]. Positive plaques were isolated and purified and recombinant pBluescript SKII plasmids were excised with the ExAssist helper phage (Stratagene). Two

Fig. 1. Immunoblots of rabbit antiserum against meningococcal protein extracts. **a**, R-70 antiserum (adsorbed with *E. coli* lysate) against total protein extracts of *E. coli* XL1 Blue; **b**, as (a) but after transformation with plasmid pSK34a; **c**, anti-Men-E2p antiserum against OMV extracts of iron-sufficient *N. meningitidis* strain SD; **d**, purified Men-E2p probed with antiserum against whole *A. vinelandii* E2p; **e**, purified Men-E2p probed with antiserum against the lipoyl domain of *A. vinelandii* E2p. *c. 70 kDa.

representative recombinant plasmids (pSK18a and pSK34a) were chosen and both strands of their cloned DNA were sequenced with an Applied Biosystems 373A DNA Sequencer. Oligonucleotide primers were synthesised by an Applied Biosystems 381A DNA Synthesiser and used for the sequencing. These primers were: M13 Forward, TTG TAA AAC GAC GGC CAG TG and M13 Reverse, CAC ACA GGA AAC AGC TAT GAC C.

Enzyme assays and Men-E2p characterisation

Plasmid pSK34a was transformed into competent TG2 *E. coli* cells, and ampicillin-resistant colonies were isolated, grown, harvested, sonicated and examined for the presence of dihydrolipoamide transacetylase activity by previously described methods [15]. At maximum expression level, the endogenous transacetylase activity was 1–2% of total activity measured in cell extracts obtained from empty vector transformed cells [15]. This was assayed spectrophotometrically at 240 nm and 25°C in a coupled assay with phosphotransacetylase (Sigma), measuring the formation of S-acetyldihydrolipoamide as described by Schwartz and Reed [16]. Dihydrolipoamide was prepared from DL-lipoamide as described by Reed *et al.* [17]. Protein concentrations were estimated with the microbiuret method [18] after precipitation with deoxycholic acid and trichloroacetic acid [19]. Bovine serum albumin was used as the standard.

Isolation of expressed Men-E2p from *E. coli*

A single colony of *E. coli* TG2 (pSK34a) was picked from a yeast-tryptone (YT) agar plate containing

>-- ORF1 ---->																		
	D	R	Y	N	V	A	V	A	A	L	A	A	L	A	E	Q	G	17
1	GAT	CGC	TAC	AAC	GTT	GCC	GTG	GCC	GCA	TTG	GCC	GCA	TTG	GCG	GAA	CAA	GGC	34
	K	V	S	K	E	T	V	Q	Q	A	I	E	K	Y	G	I	K	
52	AAA	GTC	AGC	AAA	GAA	ACC	GTT	CAA	CAA	GCC	ATT	GAG	AAA	TAC	GGC	ATC	AAA	43
	A	D	S	A	P	S	W	K	R	Stop								
103	GCC	GAT	TCA	GCT	CCT	AGC	TGG	AAA	CGC	TGA	TTGATGTTTTCAGACGGCCTGTTTGCCC							
159	CATTCCGACATCAGGCCGTCTGAAAACCGAATGCCCGAATGGTTTGTAGCAGACAAACCGTACCGATG																	
227	CCGCCTGAAGCAGCTTTCA GAC GGC ATC CAA TTG AAA AAG ATT AAA GGA ACT CAA																	
>-- ORF2 ---->																		
	M	S	I	V	E	I	K	V	P	D	I	G	G	H	E	N	V	17
282	ATG	AGT	ATC	GTA	GAA	ATC	AAA	GTC	CCC	GAT	ATC	GGC	GGT	CAC	GAA	AAC	GTC	34
	D	I	I	A	V	E	V	K	A	G	D	T	I	A	V	D	D	
333	GAC	ATC	ATC	GCC	GTA	GAA	GTT	AAA	GCG	GGC	GAC	ACC	ATC	GCC	GTT	GAC	GAC	51
	T	L	I	T	L	E	T	D	K	A	T	M	D	V	P	A	D	
384	ACC	CTG	ATT	ACA	CTG	GAA	ACC	GAC	AAA	GCC	ACG	ATG	GAT	GTG	CCT	GCC	GAT	68
	A	A	G	V	V	K	E	V	K	V	K	V	G	D	K	I	S	
435	GCG	GCC	GGT	GTC	GTG	AAA	GAA	GTA	AAA	GTC	AAA	GTC	GGC	GAC	AAA	ATC	TCC	85
	E	G	G	V	I	L	T	V	E	T	G	A	A	A	A	E	A	
486	GAA	GGC	GGC	GTA	ATT	CTG	ACC	GTT	GAA	ACC	GGT	GCC	GCC	GCC	GCC	GAA	GCC	102
	A	P	A	A	A	E	A	Q	P	E	P	A	A	A	P	A	A	
537	GCC	CCG	GCT	GCT	GCC	GAA	GCA	CAA	CCT	GAA	CCT	GCT	GCC	GCA	CCC	GCT	GCC	119
	A	G	G	A	T	V	Q	V	A	V	P	D	I	G	G	H	T	
588	GCA	GGC	GGT	GCA	ACC	GTT	CAA	GTA	GCC	GTT	CCC	GAT	ATC	GGC	GGC	CAT	ACC	136
	D	V	D	V	I	A	V	E	I	K	V	G	D	T	V	A	E	
639	GAT	GTG	GAT	GTA	ATC	GCC	GTT	GAA	ATC	AAA	GTG	GGC	GAC	ACC	GTT	GCC	GAA	153
	D	D	T	L	I	T	L	E	T	D	K	A	T	M	D	V	P	
690	GAC	GAC	ACG	CTG	ATT	ACT	TTG	GAA	ACC	GAT	AAA	GCG	ACA	ATG	GAC	GTA	CCT	170
	C	T	A	A	G	V	V	K	A	V	F	L	K	V	G	D	K	
741	TGT	ACC	GCT	GCC	GGT	GTC	GTT	AAA	GCC	GTA	TTC	TTA	AAA	GTC	GGC	GAC	AAA	187
	V	S	E	G	S	A	I	I	E	V	E	T	V	G	S	A	A	
792	GTA	TCC	GAA	GGC	TCT	GCC	ATT	ATC	GAA	GTA	GAA	ACC	GTC	GGC	TCT	GCC	GCA	204
	A	A	P	A	Q	A	A	Q	A	A	A	P	A	A	A	P	P	
842	GCA	GCC	CCT	GCT	CAA	GCC	GCT	CAA	GCT	GCC	GCA	CCG	GCT	GCC	GCT	CCG	CCT	221
	P	T	A	A	A	A	P	A	A	A	P	A	P	S	A	P	A	
893	CCG	ACT	GCT	GCC	GCC	GCA	CCC	GCC	GCC	GCG	CCT	GCA	CCT	TCT	GCA	CCT	GCC	238
	A	A	K	I	D	E	A	A	F	A	K	A	H	A	G	P	S	
944	GCT	GCC	AAA	ATC	GAC	GAG	GCC	GCT	TTC	GCC	AAA	GCA	CAC	GCC	GGT	CCT	TCC	255
	A	R	K	L	A	R	E	L	G	V	D	L	G	Q	V	K	G	
995	GCA	CGC	AAA	CTG	GCG	CGC	GAA	TTG	GGC	GTG	GAT	TTG	GGC	CAA	GTC	AAA	GGC	272
	T	G	L	K	G	R	I	M	G	D	D	I	K	A	F	V	K	
1046	ACC	GGC	TTG	AAA	GGC	CGT	ATC	ATG	GGC	GAC	GAC	ATC	AAA	GCC	TTT	GTG	AAA	289
	S	V	M	Q	G	G	A	A	K	P	A	A	A	S	A	S	L	
1097	TCC	GTG	ATG	CAG	GGC	GGC	GCG	GCA	AAA	CCT	GCC	GCA	GCC	AGC	GCA	TCT	TTG	306
	G	G	G	L	D	L	L	P	W	P	K	V	D	F	S	K	F	
1148	GGC	GGC	GGT	CTG	GAC	TTA	CTG	CCG	TGG	CCT	AAA	GTG	GAC	TTC	TCC	AAA	TTC	323
	G	N	V	E	V	K	E	L	S	R	I	K	K	I	S	G	Q	
1199	GGC	AAT	GTC	GAA	GTT	AAA	GAA	TTG	TCC	CGC	ATT	AAG	AAA	ATT	TCC	GGT	CAA	340
	N	L	S	R	N	W	V	V	I	P	H	V	T	V	H	E	E	
1250	AAC	CTG	TCC	CGC	AAC	TGG	GTT	GTG	ATT	CCC	CAC	GTT	ACC	GTA	CAC	GAA	GAA	357
	A	D	M	T	E	L	E	E	F	R	K	Q	L	N	K	E	W	
1301	GCG	GAC	ATG	ACC	GAG	CTG	GAA	GAA	TTC	CGC	AAA	CAG	CTG	AAC	AAA	GAA	TGG	374
	E	R	E	G	V	K	L	S	P	L	A	F	I	I	K	A	S	
1352	GAA	CGC	GAA	GGC	GTG	AAA	CTG	TCC	CCG	TTG	GCG	TTC	ATC	ATC	AAA	GCC	TCT	391
	V	S	A	L	K	A	F	P	E	F	N	A	S	L	D	G	D	
1403	GTT	TCC	GCG	TTG	AAA	GCA	TTC	CCC	GAA	TTC	AAC	GCC	TCA	CTG	GAC	GGC	GAC	408
	N	L	V	L	K	N	Y	F	N	I	G	F	A	A	D	T	P	
1454	AAC	CTG	GTG	CTG	AAA	AAC	TAC	TTC	AAC	ATC	GGT	TTC	GCA	GCC	GAT	ACG	CCG	425
	N	G	L	V	V	P	V	I	K	D	V	D	Q	K	G	L	K	
1505	AAC	GGC	TTG	GTT	GTT	CCC	GTC	ATC	AAA	GAC	GTG	GAT	CAA	AAA	GGC	TTG	AAA	

Fig. 2. The sequence data for cloned meningococcal DNA of pSK34a and the deduced amino-acid sequence of the ORFs. A putative ribosome-binding site for ORF2 is underlined. Stop = translational stop codon.

	Q	I	S	Q	E	L	T	E	L	S	K	K	A	R	E	G	K	442
1556	CAA	ATC	AGC	CAA	GAA	TTG	ACC	GAA	TTG	TCC	AAA	AAA	GCC	CGT	GAA	GGC	AAG	
	L	K	P	Q	E	M	Q	G	A	C	F	T	I	S	S	L	G	459
1607	CTC	AAA	CCG	CAA	GAA	ATG	CAA	GGC	GCG	TGC	TTT	ACC	ATT	TCC	AGC	TTA	GGC	
	G	I	G	G	T	G	F	T	P	I	V	N	A	P	E	V	A	476
1658	GGC	ATC	GGC	GGC	ACA	GGC	TTC	ACG	CCA	ATT	GTG	AAC	GCT	CCC	GAA	GTC	GCC	
	I	L	G	V	C	K	S	Q	I	K	P	V	W	N	G	K	E	493
1709	ATC	TTG	GGC	GTG	TGC	AAA	TCC	CAA	ATC	AAA	CCT	GTT	TGG	AAC	GGC	AAA	GAG	
	F	A	P	R	L	M	C	P	L	S	L	S	F	D	H	R	V	510
1760	TTT	GCC	CCG	CGC	CTG	ATG	TGC	CCG	TTG	AGC	CTG	TCC	TTC	GAC	CAC	CGT	GTC	
	I	D	G	A	A	G	M	R	F	T	V	F	L	A	K	L	L	527
1811	ATC	GAC	GGT	GCG	GCC	GGT	ATG	GCG	TTC	ACC	GTA	TTC	TTG	GCG	AAG	CTG	TTG	
	K	D	F	R	R	I	T	L	Stop									535
1861	AAA	GAC	TTC	CGC	CGC	ATT	ACC	TTA	TAA	AAT	AAA	ACA	TCC	CTC	TCA	AGC	AGT	

1912 CTGATAATGTTTGGATTGCTTGAGATTGATGAGTAATGGTGTAAATTCAACCTTTAAATTAATAA

>--- ORF3 --->

	L	M	G	N	F	L	Y	R	G	I	S	C	Q	Q	D	E	Q	17
1980	CTT	ATG	GGA	AAT	TTC	TTA	TAT	AGA	GGC	ATT	AGT	TGC	CAA	CAA	GAT	GAG	CAA	
	N	N	G	Q	L	K	P	K	G	N	K	A	E	V	A	I	R	34
2031	AAT	AAT	GGA	CAG	TTA	AAA	CCT	AAA	GGT	AAT	AAA	GCT	GAA	GTT	GCA	ATT	CGT	
	Y	D	G	K	F	K	Y	D	G	K	A	T	H	G	P	S	V	51
2082	TAT	GAT	GGT	AAG	TTT	AAA	TAT	GAT	GGT	AAA	GCT	ACA	CAT	GGT	CCA	AGT	GTG	
	K	N	A	V	Y	A	H	Q	I	E	T	G	L	Y	D	G	C	68
2133	AAG	AAT	GCA	GTT	TAC	GCC	CAT	CAA	ATT	GAA	ACA	GGT	CTA	TAT	GAC	GGA	TGT	
	Y	I	S	T	T	T	D	K	E	I	A	K	K	F	A	T	S	85
2184	TAT	ATA	TCT	ACG	ACA	ACA	GAC	AAG	GAA	ATT	GCC	AAG	AAA	TTT	GCA	ACA	AGT	
	S	G	I	E	N	G	Y	I	Y	V	L	N	R	D	L	F	G	102
2235	TCC	GGC	ATC	GAA	AAT	GGC	TAT	ATA	TAT	GTT	TTA	AAT	AGG	GAT	TTG	TTT	GGT	
	Q	Y	S	I	F	E	Y	E	V	E	H	P	E	N	P	N	E	119
2286	CAA	TAT	TCT	ATT	TTT	GAA	TAT	GAG	GTT	GAA	CAT	CCA	GAA	AAC	CCA	AAT	GAG	
	K	E	V	T	I	R	A	E	D	C	G	C	I	P	E	E	V	136
2337	AAG	GAA	GTA	ACA	ATC	AGA	GCT	GAA	GAT	TGT	GGC	TGT	ATT	CCT	GAA	GAA	GTG	
	I	I	A	K	E	L	I	E	I	N	Stop							146
2388	ATT	ATT	GCT	AAA	GAG	TTG	ATA	GAA	ATT	AAC	TAA	GTT	GA					

>--- ORF4 --->

	K	V	N	I	M	A	L	V	E	L	K	V	P	D	I	G	G	17
2426	AAG	GTC	AAT	ATA	ATG	GCT	TTA	GTT	GAA	TTG	AAA	GTG	CCC	GAC	ATT	GGC	GGA	
	H	E	N	V	D	I	I	A	V	E	V	N	V	G	D	T	I	34
2477	CAC	GAA	AAT	GTA	GAT	ATT	ATC	GCG	GTT	GAA	GTA	AAC	GTG	GGC	GAC	ACT	ATT	
	A	V	D	D	T	L	I	T	L	E	T	D	K	A	T	M	D	51
2528	GCT	GTG	GAC	GAT	ACC	CTG	ATT	ACT	TTG	GAA	ACC	GAT	AAA	GCG	ACT	ATG	GAC	
	V	P	A	E	V	A	G	V	V	K	E	V	K	V	K	V	G	68
2579	GTA	CCT	GCT	GAA	GTT	GCA	GGC	GTA	GTC	AAA	GAA	GTT	AAA	GTT	AAA	GTC	GGC	
	D	K	I	S	E	G	G	L	I	V	V	V	E	A	E	G	T	85
2630	GAC	AAA	ATC	TCT	GAA	GGT	GGT	TTG	ATT	GTC	GTC	GTT	GAA	GCT	GAA	GGC	ACG	
	A	A	A	P	K	A	E	A	A	A	A	P	A	Q	E	A	P	102
2681	GCA	GCC	GCT	CCT	AAA	GCC	GAA	GCG	GCT	GCC	GCC	CCG	GCG	CAA	GAA	GCC	CCT	
	K	A	A	A	P	A	P	Q	A	A	Q	F	G	G	S	A	D	119
2732	AAA	GCT	GCC	GCT	CCT	GCT	CCG	CAA	GCC	GCG	CAA	TTC	GGC	GGT	TCT	GCC	GAT	
	A	E	Y	D	V	V	V	L	G	G	G	P	G	G	Y	S	A	136
2783	GCC	GAG	TAC	GAC	GTG	GTC	GTA	TTG	GGT	GGC	GGT	CCC	GGC	GGT	TAC	TCC	GCT	
	A	F	A	A	A	D	E	G	L	K	V	A	I	V	E	R	Y	153
2834	GCA	TTT	GCC	GCT	GCC	GAT	GAA	GGC	TTG	AAA	GTC	GCC	ATC	GTC	GAA	CGT	TAC	
	K	T	L	G	G	V	C	L	N	V	G	C	I	P	S	K	A	170
2885	AAA	ACT	TTG	GGC	GGC	GTT	TGC	CTG	AAC	GTC	GGC	TGT	ATC	CCT	TCC	AAA	GCC	
	L	L	H	N	A	A	V	I	D	E	V	R	H	L	A	A	N	187
2936	TTG	TTG	CAC	AAT	GCC	GCC	GTT	ATC	GAC	GAA	GTG	CGC	CAC	TTG	GCT	GCC	AAC	
	G	I	K	Y	P	E	P	E	L	D	I	D	M	L	R	A	Y	204
2987	GGT	ATC	AAA	TAC	CCC	GAG	CCG	GAA	CTC	GAC	ATC	GAT	ATG	CTT	CGC	GCC	TAC	
	K	D	G	V	V	S	R	L	T	G	G	L	A	G	M	A	K	221
3038	AAA	GAC	GGC	GTA	GTT	TCC	CGC	CTC	ACG	GGC	GGT	TTG	GCA	GGT	ATG	GCG	AAA	
	S	R	K	V	D	V	I	Q	G	D	G	Q	F	L				235
3039	AGC	CGT	AAA	GTG	GAC	GTT	ATC	CAA	GGC	GAC	GGG	CAA	TTC	TTA	GA			

Fig. 2. (continued).

ampicillin 50 mg/L and grown overnight at 37°C in 10 ml of YT broth containing ampicillin 75 mg/L. A 100- μ l sample of this culture was used to inoculate 10 ml of the same medium which was then incubated at 37°C for 8 h. Each of six 2-L flasks with 500 ml of YT containing ampicillin 75 mg/L and IPTG 20 mg/L were inoculated with 0.5 ml of this starter culture and incubated for 16 h at 37°C in a controlled environment shaker at 300 rpm. Cells (5 g) were harvested and used for isolation of Men-E2p. The cells were suspended in 27 ml of 50 mM potassium phosphate buffer containing 2 mM EDTA, 0.1 mM phenylmethane sulphonyl fluoride (PMSF) (Merck) and 1 mM benzamidine, pH 7.0, and disrupted with a French press applying two cycles at 18 000 psi. After centrifugation for 1 h at 14 000 *g*, the supernate was adjusted to a protamine sulphate final concentration of 0.27% w/v, in which Men-E2p remained soluble. The turbid solution was centrifuged for 45 min at 14 000 *g* and the clear supernate was concentrated to 5 ml by ultrafiltration (Amicon YM100, Grace Amicon, Essebaan, The Netherlands). The concentrate was then applied to a Sephacryl S-400HR column (2.4 $\times$ 100 cm) (Pharmacia), equilibrated with 50 mM potassium phosphate buffer containing 0.5 mM EDTA, 0.1 mM PMSF and 1 mM benzamidine, pH 7.0. Enzymically active fractions were analysed on SDS-PAGE and those with pure Men-E2p contents were pooled. After concentration to 3 ml by ultrafiltration (Amicon YM100) the protein was stored in liquid nitrogen.

For immunisation, 1 mg of purified Men-E2p was electrophoresed on a preparative SDS-polyacrylamide gel. The gel was then stained for 1 min with Coomassie Brilliant Blue R250, and the protein band of interest was excised and recovered from the gel slice by electroelution in 2.5 mM Tris-HCl, 20 mM glycine, 0.35 mM SDS.

Limited proteolysis and N-terminal sequence analysis

Limited proteolysis was performed on Men-E2p in 50 ml of potassium phosphate buffer containing 0.5 mM EDTA, pH 7, with trypsin (Sigma) 0.1% w/w at 4°C for 60 min. The reaction was stopped by the addition of 1 mM PMSF and samples were subjected to SDS-PAGE.

Purified Men-E2p (400 μ g) was blotted from SDS-polyacrylamide gels on to Immobilon (Millipore, Watford) and used for N-terminal amino-acid analysis, with an Applied Biosystems 475 Protein Sequencer.

Results

Cloning and sequence analysis

Screening of *c.* 50 000 plaques for specific protein expression identified 100 positive plaques. Most

plaques were recognised by the R-70 antiserum under both native and denatured conditions. Twenty-five of the most strongly positive plaques were rescued and analysed by immunoblotting for protein expression before and after IPTG-induction. Of the examined plasmids, pSK34a (3.1-kb insert) showed the highest level of expression of cloned protein of *c.* 70 kDa (Fig. 1, lane b). This expression was not affected by IPTG stimulation. Plasmid pSK18a (1.5-kb insert) also showed strong expression of a protein reactive with R-70, but with lower molecular mass (*c.* 65 kDa, not shown).

Both plasmids were restriction-mapped and both of their strands were fully sequenced (Fig. 2), EMBL accession number X82637. The main features of the analysed DNA sequence are shown in Fig. 3. Four open reading frames (ORFs) were predicted from the sequence analysis. The deduced amino-acid sequence of ORF1, ORF2 and ORF4 showed a high degree of homology with the amino-acid sequences of *E. coli* pyruvate dehydrogenase (E1p), dihydrolipoyl acetyltransferase (E2p) and dihydrolipoyl dehydrogenase (E3), respectively. These are components of the pyruvate dehydrogenase complex (PDHC). The deduced sequence of ORF2 showed amino-acid identity with the E2 component of PDHC, and the 2-oxoglutarate dehydrogenase complexes of a number of prokaryotic and eukaryotic cells, including *E. coli*, *Azotobacter vinelandii*, *Alcaligenes eutrophus* [20–22], yeasts, rats and man. Fig. 4 shows sequence alignment of meningococcal E2p (Men-E2p) with the E2p of PDHC of the other three gram-negative species.

ORF1 and ORF4 were incomplete genes, being part of larger coding regions. Indeed, subsequent sequence data (not published) confirmed that ORF1 is part of an 887 amino-acid long reading frame (included within X82637). The deduced sequence of ORF4 was almost identical (>99% homology) to the N-terminal sequence of the previously sequenced meningococcal OMP64 (Accession No. X77920) [23]. However, ORF3 failed to show significant homology with any described DNA or protein sequence. The most closely related protein was yeast mitochondrial precursor proteins import receptor and heat shock protein homologue (with *c.* 90 amino acid overlap, 22% homology). The DNA sequence of ORF3 showed homology with a number of unrelated genes, such as a region of the replication origin of *Bacillus subtilis* and eukaryotic ATP synthase gamma-subunit. An ORF of this size and position (between E2p and E3 components of PDHC) is thus far only found in the gene cluster encoding PDHC of *Al. eutrophus* [22]. Unlike the latter, the base composition of ORF3 deviates considerably from that of the other ORFs.

Finally, the deduced sequence of none of the ORFs within pSK34a showed significant homology with the

Fig. 3. Schematic representation of a partial restriction map and computer-predicted features of the cloned DNA of pSK18a and pSK34a plasmids. ORF, open reading frame. Numbers indicate distance (in bp) from the 5' end. The domain structure of Men-E2p, as deduced from the sequence homology data with other E2 sequences, is also shown. LD-1 and LD-2, lipoyl domains 1 and 2; BD, E1/E3 binding domain; HR, hinge region.

recently published sequences of meningococcal or gonococcal FrpB protein [8, 24].

Functional characterisation of meningococcal E2p (Men-E2p)

Plasmid pSK34a, which contained the complete ORF2, was chosen for expression of the ORF2 product (Men-E2p) in *E. coli* TG2. Men-E2p was expressed and purified and its properties were examined.

The E2p activity of the Men-E2p-expressing *E. coli*

TG2 was low in the exponential growth phase (at 8 h growth); however, it increased steadily towards the end-log and stationary phase and was optimal after 16 h. Therefore, cells were harvested at this stage, when the E2p activity was 4 U/mg (Table 1). This is similar to that of *A. vinelandii* E2p expressed in the same *E. coli* strain (4.4 U/mg) [25], except that the cell yield was only 50% for Men-E2p.

When examined by SDS-PAGE, the Men-E2p-expressing *E. coli* TG2 showed a strongly stained protein band at *c.* 70 kDa (Fig. 5), as expected for an E2p possessing two lipoyl domains. The corresponding

Table 1. Purification of meningococcal E2p from *E. coli* TG2 (pSK34a)

Sample	Volume (ml)	Protein content (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)
Crude extract	27	483	4.0	1932	100
Protamine sulphate	29	268	6.8	1816	94
S400HR eluate	3	21	58.2	1243	64

Fig. 4. Structure-based sequence alignment of E2p from *A. vinelandii* (A.vin), *E. coli* (E.col), *Al. eutrophus* (A.eut) and *N. meningitidis* (N.men). The structural comparison of the first lipoyl domain (A) is based on the NMR data obtained for the *A. vinelandii* N-terminal lipoyl domain [30]. For the E1/E3 binding domain (B), the NMR data of the E3 binding domain from *E. coli* E2o [29] have been used. The structural comparison of the catalytic domain (C) is based on the co-ordinates of the catalytic domain from *A. vinelandii* E2p [28]. Secondary structure is indicated by the letters h (helix) and e (sheet) for each of these domains. †The catalytically important residues. *The residues involved in intertrimer interaction of the catalytic domain.

	Hinge			<Catalytic domain	
	370	380	390	400	410
A.vin	VQAYVKAMMQKAKEAPAAGAASGAG-----	IPPIPPVDFAKYGEIEEVPMT			
E.col	AIKRAEAAPAATGGGIPG-----	ML.W.K...S.F.....ELG.			
A.eut	..G...GVMMSGQAAAPAQAAAAGAGGGELGLL.W.K...TRF..V.SKALS.				
N.men	IK.F..SVMQGGAAKPAAASASLGGGLD--LL.W.K...S.F.NV.VKELS.				
	hhhhh			eeee	Ca
	420	430	440	450	460
				* * *	
A.vin	LMQIGATNLHRSWLNVPVHTQFESADITELEAFRVAQKAVAEEKAG--VKLTV				
E.col	IQK.SGA..S.N.VMI...H.DKT.....KQ.NEE.A.RKLD..I.P				
A.eut	IKK.SGA...N.VMI.NHD...E.....LQLNKEN.S--I.V.M				
N.men	IKK.SGQ..S.N.VVI.VH...EA.M...E..KQLNKEW.RE--...SP				
	hhhhhhhhhhhhhhhhhhhh	eeeeeee	hhhhhhhhhhhhhhhhhhhh	h	
	CH1	Cb	CH2		
	470	480	490	500	510
A.vin	LPLLLKACAYLLKELPDFNSSLAPSGQALIRKKYVHIGFAVDTPDGLLV				
E.col	VVFIM..V.AA.EQM.R....SED..R.TL...IN..V.....N..V...				
A.eut	.AFMI..TVAA..KF.N..A..DGDN--.VL...FN....A...N..V...				
N.men	.AFII..SVSA..AF.E..A..DGDN--.VLKN.FN....A...N..V...				
	hhhhhhhhhhhhhhhhhhhh	eee	eeeeee	eeeeee	eeee
	CH3	Cc	Cd	Ce	Cf
	520	530	540	550	560
		****			†
A.vin	IRNVDQKSLQLAAEAAELA EKARSKKLGADAMQGACFTISSLGHIGGTAF				
E.col	FKD.NK.GIE.SR.LMTISK...DG..T.GE...G.....I.GL.T.H.				
A.eut	.KDA.K.GV.EISQ.MS...KL..DG..KP.Q...G..S.....GL...Y.				
N.men	.KD....G.K.ISQ.LT..SK...EG..KPQE.....G....G.				
	ee	hhhhhhhhhhhhhhhhhhhh		eeeeee	eeee
		CH4		Cg	Ch
	570	580	590	600	610
					† †
A.vin	TPIVNAPEVAILGVSKASMQPVWDGKAFQPRMLPLSLSYDHRVINGAAAA				
E.col	A.....SA.E...N..E.V.....F.....D..DG.				
A.eut	...I....M..C.SYQK.....Q.A...T.....W.....D..E.R				
N.men	...G....G....C.SQIK...N..E.A....C....F.....D...GM				
	eee	eeeeee		eeeeeeee	hhhhh
		Ci		Cj	CH5
	620	630			

A.vin	RFTKRLGDLLADIRAILL				
E.col	..ITIINNT.S...RLVM				
A.eut	FN.-YF.Q....F.RI..				
N.men	...VF.AK..K.F.RIT.				
	hhhhhhhhhhhh	hhhhh			
		CH6			

Fig. 4. (continued).

host proteins, E2o containing a single lipoyl domain and E2p containing three lipoyl domains, show bands in SDS gels at *c.* 56 kDa and *c.* 83 kDa, respectively [26].

The steps used in the purification of biologically active Men-E2p were similar to those used for the

isolation of E2p from *A. vinelandii* [25]. The isolated Men-E2p did not contain *E. coli* E3, indicating that the E3 component of *E. coli* PDHC did not interact with Men-E2p. This made the purification procedure for Men-E2p much simpler than that for *A. vinelandii* E2p. The purified Men-E2p had a specific E2p activity of 58 U/mg (Table 1), which is similar to that of

Fig. 5. Purification of meningococcal E2p expressed in *E. coli* TG2 (pSK34a). Samples from different stages of the isolation procedure were subjected to SDS-PAGE. Lane **M**, low mol. wt markers (Sigma) (kDa); **1**, cell-free extract; **2**, supernate after protamine sulphate precipitation; **3**, concentrated supernate after protamine sulphate precipitation; **4**, pooled from Sephacryl S-400HR.

purified *A. vinelandii* E2p (54–79 U/mg) [25]. The sequence of the first 10 N-terminal amino acids of the purified Men-E2p was determined, and shown to be (M)SIVEIKVPDI, confirming the predicted sequence

of the mature protein. The initiating methionine was only partially removed.

Immunochemical characterisation of Men-E2p

Rabbit antiserum was raised against the purified Men-E2p (extracted from preparative SDS-polyacrylamide gels) and compared in immunoblots with the R-70 antiserum. Both antisera reacted strongly with the purified Men-E2p (Fig. 6, lanes 3 and 5).

The predicted amino-acid sequence of Men-E2p shows the presence of several lysine residues in all linker regions and, therefore, it was predicted that limited proteolysis with trypsin would yield a single lipoyl domain (c. 16 kDa), a catalytic domain (c. 30 kDa), a lipoyl-lipoyl di-domain (c. 36 kDa) and a lipoyl-E1/E3 binding-catalytic tri-domain (c. 54 kDa). SDS-PAGE analysis (Fig. 6) showed that all the predicted fragments were present (lane 2), with the lipoyl-E1/E3 binding-catalytic tri-domain, the lipoyl-lipoyl di-domain and the catalytic domain being the main fragments, and the single lipoyl domain being a minor product. These fragments are also present in the undigested E2p (lane 1) albeit in minor amounts. They probably originate from proteolytic activity in the TG2 cells. Rabbit anti-Men-E2p serum (Fig. 6, lanes 5 and 6) reacted strongly with all the fragments (i.e., all the domains), whereas R-70 antiserum (lanes 3 and 4) showed a stronger reaction against fragments which contained the lipoyl domain and a much weaker reaction against the catalytic domain.

Purified Men-E2p also reacted with antisera previously raised against the native dihydrolipoyl acetyltransfer-

Fig. 6. Coomassie Brilliant Blue-stained SDS-polyacrylamide gel (lanes **M**, **1** and **2**) and immunoblots (lanes **3**–**6**) of purified meningococcal E2p treated with trypsin. Lane **M**, low mol. wt markers (kDa); **1**, **3**, **5**, purified E2p; **2**, **4**, **6**, E2p digested with trypsin; **3** and **4**, blot incubated with R-70 antiserum; **5** and **6**, blot incubated with anti Men-E2p antiserum. Fragments visible: **a**, intact E2p; **b**, lipoyl-E1/E3 binding-catalytic tri-domain; **c**, lipoyl-lipoyl di-domain; **d**, catalytic domain; **e**, single lipoyl domain. Equal amounts of E2p were added to each lane.

ase of *A. vinelandii* (Fig. 1, lane d) and that raised to its lipoyl domain (Fig. 1, lane e). This further indicated that E2p is highly conserved across species in terms of immunoreactivity. Also, rabbit anti-Men-E2p antiserum cross-reacted with total proteins of *A. vinelandii*, *E. coli* and *N. gonorrhoeae* (not shown). Men-E2p failed to react with a murine monoclonal anti-FrpB antibody, MAb-M70 (not shown), indicating that this protein is different from the *c.* 70-kDa iron-regulated FrpB. This was further confirmed when rabbit anti-Men-E2p serum reacted equally well with the OMVs obtained from meningococci (strain SD) grown under iron-sufficient (Fig. 1, lane c) and iron-restricted conditions (not shown).

It was of interest to determine whether or not Men-E2p is immunogenic following meningococcal infection. Rabbit antisera, raised against live meningococcal strains SD (B:15:P1.16) (the strain used for Men-E2p cloning), AS (A:4:P1.7) and EB (29E), and the commensal strain, *N. lactamica*, reacted strongly with purified Men-E2p in immunoblots (Fig. 7, lanes a–d). Murine antisera raised against live meningococcal strains SD and B16B6 (B:2a:P1.2) also reacted with Men-E2p (Fig. 7, lanes e and f). No reaction was detected when rabbit or murine pre-immune sera were used (not shown).

Antibodies to purified Men-E2p were also detected in human convalescent sera obtained from three patients who had recovered from meningococcal diseases caused by three different strains, including strain SD, JB (C:NT) and GLD (C:2a:P1.10), and one patient who recovered from gonococcal pelvic inflammatory disease (Fig. 6, lanes g–j). It is important to note that normal human serum, obtained from a healthy individual with no history of meningococcal or gonococcal infection, also showed a very weak

reaction with Men-E2p (not shown because the band was lost on photoreproduction).

Discussion

The pyruvate dehydrogenase complex of many gram-negative bacteria contains three enzymic components, pyruvate dehydrogenase (E1p), dihydrolipoyl acetyltransferase (E2p) and dihydrolipoyl dehydrogenase (E3). These are encoded by three genes (*aceE*, *aceF* and *lpd*) [27]. Thus far, three dihydrolipoyl acetyltransferases from gram-negative organisms have been sequenced and characterised [20–22]. The E2p from *N. meningitidis* (Men-E2p) characterised in this study presents the fourth example. Men-E2p has a domain structure that compares very well with that of other E2ps. From the sequence analysis it was clear that it contains two N-terminal lipoyl domains, an E1/E3 binding domain and a catalytic domain. The domains are separated by hinge regions rich in alanine, proline and charged residues. Men-E2p shares a high degree of homology with the E2p of the other three bacteria, with 50–55% sequence identity in each of the domains. Unlike *E. coli* and *A. vinelandii* enzymes, Men-E2p lacks a third lipoyl domain. Instead, and similar to the PDHC of *Al. eutrophus* [22], meningococci seem to express an additional lipoyl domain on the N-terminus of the E3 component. The function of this lipoyl domain in the overall catalysis by the complex remains an interesting aspect for future research. Another interesting feature is the presence of an additional reading frame, ORF3, between the coding regions of E2p and E3. Expression of a protein that corresponds to ORF3 (16.5 kDa) has not been observed. A similar ORF has been identified only in *Al. eutrophus*. However, unlike the latter, the codon usage of the meningococcal ORF3 is very different from the other

Fig. 7. Immunoblots of purified Men-E2p probed with antisera to live strains: **a–d**, probed with rabbit antiserum to live *N. meningitidis* strains SD, AS, EB and live *N. lactamica*, respectively; **e–f**, probed with murine antiserum to strains SD and B16B6; **g–j**, probed with human convalescent sera from patients infected with *N. meningitidis* strains SD, JB, GLD and *N. gonorrhoeae*, respectively. * *c.* 70 kDa.

coding regions so it is doubtful that it codes for an additional protein.

The sequence of four highly similar E2p proteins from gram-negative organisms, together with the availability of structures of each of the domains [28–30], allows a detailed structure-based comparison (Fig. 4). The sequence around the lipoyl-lysine residues, Lys³⁹ and Lys¹⁵⁶ (*A. vinelandii* numbering throughout), is strongly conserved. These residues are located in a type I turn connecting beta strands Ad and Ae. The extent of lipoylation has not been analysed. Strong conservation is also observed in the E1/E3 binding domain, especially around helix BH1. This helix is thought to be involved in E3 binding [31]. Despite strong sequence conservation, the affinity of *E. coli* E3 for Men-E2p must be weak as this enzyme was not associated with Men-E2p during purification. In contrast, it was found that E3 from *A. vinelandii* bound strongly to Men-E2p (not shown) and weakly to *E. coli* E2p [32]. The reason for these differences in affinity is not obvious from a sequence comparison of the binding domain alone. In the catalytic domain, the important catalytic site residues Ser⁵⁵⁸ and His⁶¹⁰ are, as predicted, conserved. *A. vinelandii* E2p is the only known E2p that has an Asn⁶¹⁴, this being an Asp in the other sequences. On the basis of mutagenesis experiments, it has been suggested recently that charge neutralisation of Asp⁶¹⁴ is required for an optimal catalytic function of the nearby His⁶¹⁰ [33]. Modelling experiments indicated that in *E. coli* E2p charge neutralisation might result from salt bridge formation between Arg⁴⁸³ and Asp⁶¹⁴. In the sequences from *Al. eutrophus* and *N. meningitidis*, Arg⁴⁸³ is replaced by Asp and Glu, respectively, which indicates that the formation of a salt bridge, as in *E. coli*, is unlikely.

Another interesting observation from the sequence comparison concerns the stability of the E2p core. In *A. vinelandii*, this core dissociates into trimers upon binding of the peripheral components, while the other E2p cores remain cubic. The intertrimer interaction is of hydrophobic character and the interface is formed by the C-terminal helix CH6, the N-terminal part of helix CH4 and one face of helix CH2 (residues indicated by asterisk in Fig. 4). In the Men-E2p sequence Ala⁶³⁴ of helix CH6 is replaced by Arg and Val⁴⁵⁶ by Glu. Modelling experiments show that these residues can form a salt bridge, thus causing additional stabilisation of the cubic core. Mutagenesis experiments are underway to verify this hypothesis.

The predicted sequence of the lipoyl domain from *N. meningitidis* E3 (deduced from the ORF4 amino-acid sequence) is almost identical to that of the N-terminal lipoyl domain of Men-E2p. We have recently shown that R-70 and rabbit anti Men-E2p antisera cross-react with meningococcal E3 (unpublished observations). E3 seems most likely to be the same protein as the meningococcal OMP64 characterised by Silva *et al.*

[23], which migrates as a *c.* 70-kDa protein in SDS-polyacrylamide gels. Therefore, it is likely that this protein co-migrates, and hence co-purifies, with FrpB and Men-E2p. The observation that R-70 antibodies react more strongly with the lipoyl domain than with other epitopes from Men-E2p, adds to the possibility that the R-70 antibodies might react primarily with the E3 component and Men-E2p through cross-reactivity of the N-terminal lipoyl domain.

Silva *et al.* [23] suggested that OMP64 is an immunogenic OMP, likely to be surface exposed and, therefore, suitable for vaccine preparation. It is difficult to see a physiological role for E3 or the complex in the outer membrane. E3 might be expressed in excess to the amount required for binding to the multi-enzyme complex, for example, under regulation by ORF3. Free or complex-bound dihydrolipoyl dehydrogenase has been described as associated with the plasma membrane in some organisms [34–36], but unless an outer membrane localisation is verified, for example, by immunogold labelling of whole cells or cell sections, a contamination of the OMV preparation by other cell components cannot be excluded. The expression of meningococcal E3 in *E. coli* and its purification is being undertaken for further analysis of this question.

E2p is highly conserved across prokaryotic and eukaryotic species and it is interesting to note that Men-E2p is immunogenic in animals when used as purified protein or as a component of live organisms. More interestingly, patients who recovered from natural meningococcal or gonococcal disease also responded to Men-E2p, with cross-reactive antibodies. It is not known whether these antibodies are against conserved parts of Men-E2p which might be shared with man. If so, this will pose the risk of breaking immune tolerance and causing autoimmune disease. Indeed, patients with primary biliary cirrhosis produce antibodies directed against the lipoyl-lysine region of the lipoyl domains of all mammalian acyltransferases [37].

Silva *et al.* [23] found that antisera from vaccinees immunised with a Cuban vaccine (Va-mengoc-BC) [3] contained antibodies to OMP64 (i.e., probably E3). This indirect evidence indicates that the vaccine contains E3 protein. The presence of Men-E2p in this vaccine is also possible, considering that, at least during OMV preparation under non-denaturing conditions, E2p and E3 molecules would be expected to remain interactive and, therefore, co-purify. Similarly, the possible presence of Men-E2p or E3 in the Norwegian OMV-based vaccine preparation cannot be excluded, as it is known that this vaccine, like the Cuban preparation, contained the iron-regulated FrpB [38, 39]. It is not known whether patients with a history of neisserial infection, or vaccinees, are more likely to develop autoimmune disease than the normal

population. However, normal individuals are exposed to common respiratory neisseriae. This may explain why the normal human serum examined contained antibodies against Men-E2p, although they were of either low quantity or affinity. Clearly, much better understanding of the immunochemistry of, and the human immune response to, meningococcal OMV components is required before a safe and effective vaccine can be developed.

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