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A mycoplasma high-affinity transport system and the *in vitro* invasiveness of mouse sarcoma cells

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FS9 mouse sarcoma cells were previously shown to be highly invasive when confronted with chicken heart fibroblasts using Abercrombie's confronted explant technique. This invasion could be inhibited by addition to the assay of Fab fragments of a monoclonal antibody directed against p37, a protein associated with the surface of FS9 cells. We have cloned and sequenced the gene for p37. We show that it originates from *Mycoplasma hyorhinis* and that UGA is a tryptophan codon in this organism. We present evidence that the p37 gene is part of an operon encoding two additional proteins which are highly similar to components of the periplasmic binding-protein-dependent transport systems of Gram-negative bacteria, and we suggest that p37 is part of a homologous, high-affinity transport system in *M.hyorhinis*, a Gram-positive bacterium. We discuss the influence of p37 and *M.hyorhinis* on contact inhibition of locomotion of mammalian cells.

Key words: genetic code/*in vitro* invasiveness/mycoplasma/transport system

Introduction

Contact interactions between living cells in culture mimic aspects of malignant invasiveness (Abercrombie, 1979; Paddock and Dunn, 1986; Parish *et al.*, 1987). The confronted explant test has been used to assay cellular invasion *in vitro* (Abercrombie, 1979). Using this system we identified a 37-kd protein (p37) on the surface of FS9 mouse fibrosarcoma cells that is involved in their invasive behaviour (Steinemann *et al.*, 1984a,b; Parish *et al.*, 1987). Monovalent antibodies (Fab) derived from polyclonal or monoclonal antibodies directed against p37 inhibited the invasion of chicken heart fibroblasts by the fibrosarcoma cells and normal heterotypic contact inhibition of locomotion was restored. We recently discovered that p37 appeared on other cell lines when these were incubated with the culture supernatant of the fibrosarcoma cells (C.Schmidhauser *et al.*, in preparation). The appearance of p37 was correlated with increased invasivity of the cells *in vitro* and this could again be inhibited by adding anti-p37 Fab to the system. Addition of the anti-mycoplasma compound BM-cycline to the fibrosarcoma cells resulted in the loss of p37 and a reduction in the invasive behaviour of the cells *in vitro*. These results suggested p37 is either a mycoplasma protein or is induced in the animal cells by mycoplasma infection.

We wished to isolate the relevant gene with the aim of obtaining clues of the function of p37 and the mechanisms underlying its remarkable effects on cell behaviour. This paper describes the cloning and sequencing of the p37 gene. We found the gene occurs in *Mycoplasma hyorhinis* and is part of an operon coding for two additional proteins. These proteins have structural similarity to the components of periplasmic binding-protein-dependent transport systems of Gram-negative bacteria, suggesting that p37 is part of a high-affinity transport system in *M.hyorhinis*, a Gram-positive bacterium. This represents the first evidence for such a system in Gram-positive bacteria.

Results

Isolation and N-terminal sequence determination of p37

We used the following strategy to clone the gene coding for p37. We first isolated p37 and sequenced its N terminus. This allowed us to synthesize oligonucleotides coding for part of the N-terminal sequence which could then be used as probes to screen DNA libraries.

To isolate p37 we prepared large amounts of the monoclonal antibody DD9, which specifically recognizes p37 (Steinemann *et al.*, 1984a,b), from ascites fluid and bound it to a solid support for use in immunoaffinity chromatography. Solubilized proteins from whole FS9 cells were run over this column and bound proteins eluted. Fractions containing proteins were run on SDS-polyacrylamide gels and stained with Coomassie blue. p37 was cut out from the gels, eluted and concentrated. The isolated protein runs as

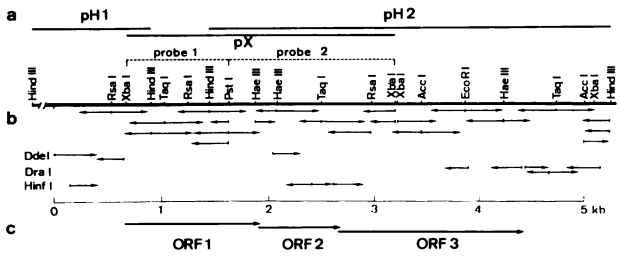
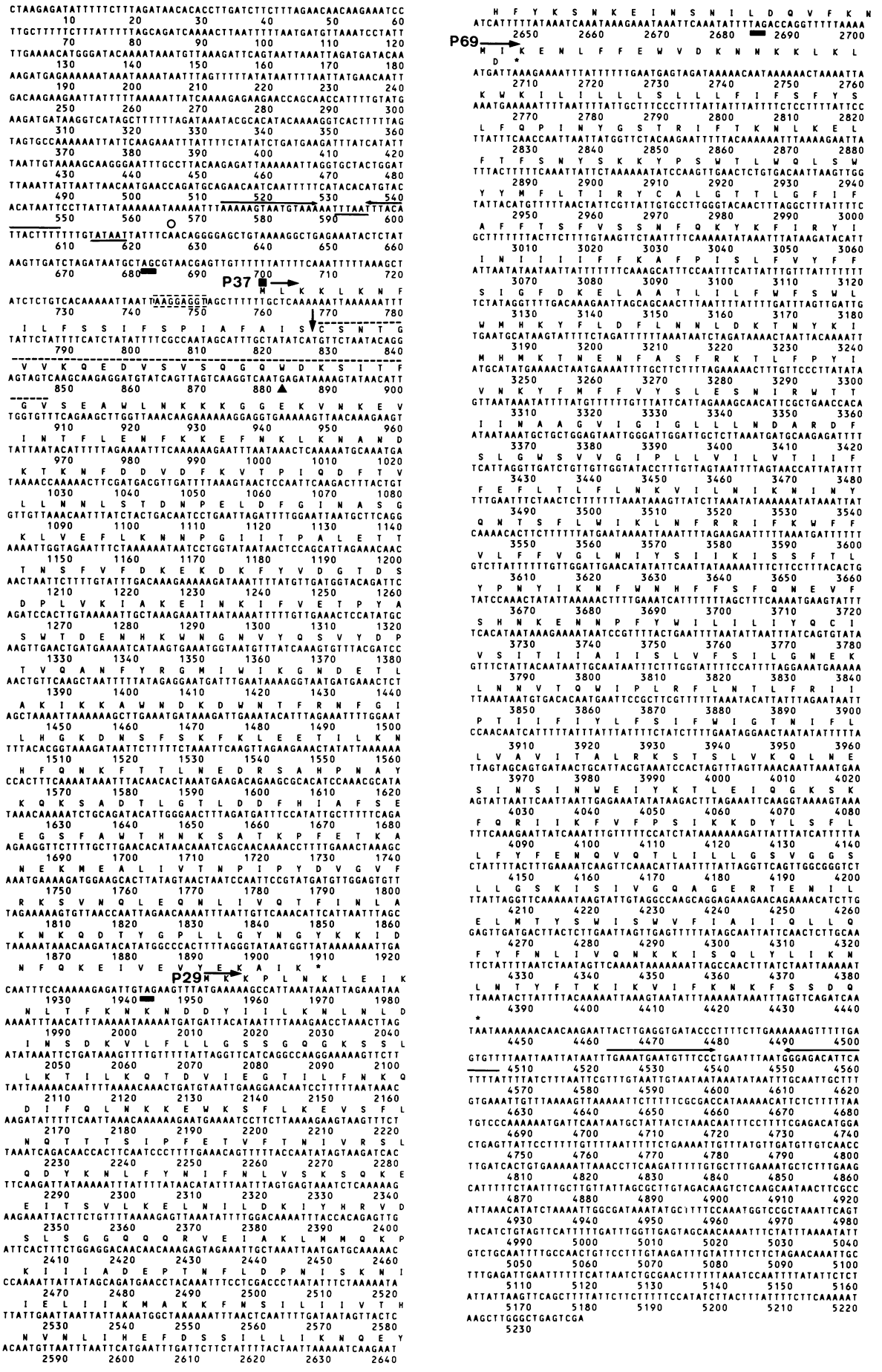


Fig. 1. Physical map and sequencing strategy of the 5.2-kb genomic DNA fragment containing ORF 1, ORF 2 and ORF 3. (**a**) The three clones from the *Hin*dIII (pH1 and pH2) and from the *Xba*I (pX) libraries are drawn in relation to the corresponding restriction map. Dashed lines denote the restriction fragments used as probes for the Southern analysis. (**b**) The sequencing strategy is indicated by arrows. For the majority of the cloned DNA, its sequence has been deduced from both strands in duplicate. All restriction sites were sequenced over. Restriction sites not included in the map but used for subcloning are listed on the left. (**c**) Arrows indicate position and transcriptional direction of ORFs 1, 2 and 3.


P37
P69
P29

a single band on SDS gels and is recognized by the DD9 monoclonal antibody on an immunoblot (not shown). Microsequencing from the N terminus yielded the first 27 amino acids: XSNTGVVKQEDVSVSQGQ(H,W,C)DKSITFGV. The first sequencing reaction cycle gave no signal, which might imply the presence of a cysteine at that position, since this amino acid cannot be detected with the method used. Amino acid 19 was ambiguously determined to be tryptophan, cysteine or histidine. We synthesized a mixture of all 64 possible antisense 17-mer oligonucleotides corresponding to amino acids 7–12 (VKQEDV): 5′AC(A,G)-TC(T,C)TC(T,C)TG(T,C)TTNAC.

Cloning

Since our initial screening of FS9 cDNA libraries was negative, we decided to screen a genomic library prepared from the contaminating mycoplasmas. However, we were not able to isolate mycoplasmas from FS9 culture supernatant in biochemically detectable quantities. Therefore we isolated DNA from the cytoplasmic fraction of FS9 cells, reasoning that genomic DNA of contaminating mycoplasmas must be included. Southern analysis of this DNA cut with *Hin*dIII or *Xba*I showed bands of 1.7 kb and 2.5 kb respectively that hybridized with the oligonucleotide mixture (not shown). Two DNA libraries were prepared in the plasmid pUC 12 using *Hin*dIII- and *Xba*I-cut DNA respectively. Screening with the 17-mer oligonucleotide mixture resulted in positive clones in both libraries. All the positive clones in the *Hin*dIII library had the same restriction map and they were designated pH1. The same was true for the positive clones in the *Xba*I library, and they were designated pX. pX was then used to isolate an overlapping clone in the *Hin*dIII library, which was named pH2. Figure 1 shows a restriction map of the stretch of DNA these clones cover and the sequencing strategy used to determine their sequence.

p37 is a protein from a prokaryote with a different genetic code

Figure 2 shows the sequence of the 5.2-kb stretch of DNA we determined. It is composed of 76% A and T. When translated in the reading frame labelled with p37 in Figure 2 (ORF 1), an amino acid sequence compatible with the N-terminal protein sequence is obtained (between nucleotide positions 827 and 907, dashed line above sequence in Figure 2). However, there is a TGA stop codon at the position corresponding to amino acid 19 of the N-terminal sequence (nucleotide position 881, marked with a filled triangle in Figure 2). This amino acid was ambiguously determined by N-terminal sequencing to be cysteine, histidine or tryptophan. Since the amino acid sequence following this stop codon is identical with the N-terminal sequence obtained by protein sequencing, we conclude that TGA is not a nonsense codon but codes for an amino acid, most likely for tryptophan, as it does in *M.capricolum* (Yamao *et al.*, 1985). At the position corresponding to the N-terminal amino acid (nucleotide 827), the cysteine codon TGT is found, compatible with the protein sequence data. However, TGT cannot be the translational initiation codon. Twenty-three amino acids upstream from this cysteine there is a TTG leucine codon (nucleotide position 758, marked with a filled square in Figure 2), preceded by potential Shine–Dalgarno sequence (Shine and Dalgarno, 1974; broken box in Figure 2). TTG is known as a translational initiation codon in several *Escherichia coli* genes (Mackie, 1981; Young *et al.*, 1981; Roy *et al.*, 1983). This TTG seems the only likely start codon between the first upstream in-frame stop codon (nucleotide position 680, marked with a thick bar in Figure 2) and the amino-terminal cysteine of p37. We therefore assume that p37 is synthesized as a presursor protein. The sequence of the 23 amino acids preceding the cysteine have the features of a typical prokaryotic signal peptide for protein export (Emr *et al.*, 1980). This is compatible with the fact that p37 is accessible in living cells to antibodies directed against it, (Steinemann *et al.*, 1984a,b; and unpublished results). Provided that TGA is read as tryptophan, ORF 1 codes for a protein of 403 amino acids including the signal peptide of 23 amino acids. Without the signal sequence, the protein is 380 amino acids in length and has a mol. wt of 43.5 kd. The apparent mol. wt of 37 kd reported by Steinemann *et al.* (1984a,b) seems to be an underestimation, since in our hands the purified protein runs on SDS gels with an apparent mol. wt of ~42 kd. Furthermore, the deduced amino acid composition corresponds well to that obtained from the isolated p37 (data not shown). We conclude from this analysis that we have cloned the gene coding for p37, that this gene originates from a prokaryote in which TGA is not a nonsense codon but is probably read as tryptophan, and that p37 seems to be synthesized as a precursor protein with a signal sequence which is processed.

Figure 4a shows the hydropathy plot of p37. With the exception of the signal peptide, it is a hydrophilic protein with no obvious membrane-spanning domains which could anchor it to the membrane. However, it is interesting to note that the N-terminal sequence C–S–N of the mature protein fits the N-terminal consensus sequence of bacterial lipoproteins (reviewed in Wu, 1987; Weyer *et al.*, 1987; Yamaguchi *et al.*, 1988). In these proteins the N-terminal cysteine is modified into a lipo amino acid which is thought to anchor them to the membrane. Thus it seems possible that p37 is attached to the membrane by the same mechanism.

Evidence that the p37 gene is part of an operon coding for two additional hypothetical proteins, p29 and p69

Assuming that TGA codes for tryptophan, the DNA sequence reveals, in addition to ORF 1, two large open reading frames (ORF 2 and 3) on the same strand (Figure 1). ORF 2 slightly overlaps the 3′ end of ORF 1 and, in turn, is followed by a third ORF slight overlapping it (Figure 1). We reasoned that three so closely spaced ORFs might belong to a single operon, thus implying a possible functional connection between the corresponding proteins.

Fig. 2. Nucleotide sequence of the 5.2-kb genomic DNA fragment and the presumed amino acid sequences of p37, p29 and p69. The proposed initiator codon TTG for p37 at position 758 is marked with a filled square above the sequence. The TGA codon at position 881, most likely read as tryptophan, is labelled with a filled triangle below the sequence. The hypothetical Shine–Dalgarno sequence from position 743 to 750 is boxed. The dashed line indicates the N-terminal protein sequence obtained by microsequencing techniques. The vertical arrow labels the site of processing of the p37 precursor protein. The first in-frame upstream stop-codons of ORFs 1, 2 and 3 are labelled with broad horizontal bars. A potential promoter region is marked by two bars beneath the −10 and −35 boxes. Position 625, assigned to be the potential initiator site of transcription, is labelled by an open circle. The horizontal arrows at bases 573–609 and 4522–4565 indicate the regions of dyad symmetry.

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P29       2  KKPLNKLEIK NL--TFKNKN DDYIILKNLN LDINSDKVLF LLGSSGQGK-
OppD     12  QPANVLLEVN DLRVTFATPD GDVTAVNDLN FTLRAGETLG IVGESGSGKS
MalK      1  -------MAS VQLQNVTKAW GEVVVSKDIN LDIHEGEFVV FVGPSGCGKS
HisP      1  ----MMSENK LHVIDLHKRY GGHEVLKGVS LQARAGDVIS IIGSSGSGKS
PstB      1  MSMVETAPSK IQVRNLNFYY GKFHALKNIN LDIAKNQVTA FIGPSGCGKS
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P29      49  -SSL--LK--  ---TILKQT- DVIEGTILF- NK-QDIFQLN KKE----WKS
OppD     62  QSRLR-LM--  ---GLLATN- GRIGGSATF- NG-REILNLP ERELNT-RRA
MalK     44  -TLLRMIA--  ---GLETITS GDLFI-GEK-  ---RMNDTP- -PAE----R-
HisP     47  -TFLRCIN--  ---FLEKPSE GAIIVNGQNI NLVRDKDGQL KVADKNQLRL
PstB     51  -TLLRTFNKM FELYPEQRAE GEILLDGDNI ---------- -LTNSQDIAL
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P29      84  --FLKEVSFL NQTTTSIPFE TVFTNIVRSL QDYKNLFYNI FNLVSKSQKE
OppD    102  --EQISMIFQ DPMTSLNPYM RVGEQLMEVL MLHKGM--SK AEAFEESVRM
MalK     76  --G-VGMVFQ SYA--LYPHL SVAEN-MSFG LKPAGA--KK EVINQRVNQ-
HisP     91  LRTRLTMVFQ HFN--LWSHM TVLENVMEAP IQVLGL--SK HDARERALKY
PstB     89  LRAKVGMVFQ KPT--PFP-M SIYDNIAFGV RLFEKL--SR ADMDERVQWA
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P29     132  EIT-SVLKEL NILDKIVHRV DSLSGGQQQR VEIAKLMMQK PKIIIADEPT
OppD    148  LDA-VKMPEA RKRMKMYPH- -EFSGGMRQR VMIAMALLCR PKLLIADEPT
MalK    117  V-A--EVLQL AHLLDRKPK- -ALSGGQRQR VAIGRTLVAE PSVFLLDEPL
HisP    137  L-A--KVGID ERAQGKYPV- -HLSGGQQQR VSIARALAME PDVLLFDEPT
PstB    134  LTKAALWNET KDKLHQSGY- -SLSGGQQQR LCIARGIAIR PEVLLLDEPC
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P29     181  NFLDPN--IS -KNI-IELII KMAKKFNSIL IIVTHNVNLI HEFDSSILLI
OppD    195  TALDVT--VQ -AQI-MTLLN ELKREFNTAI IMITHDLGVV AGICDKVLVM
MalK    162  SNLDAALRVQ -MRIEISRLH KRLGR--TMI Y-VTHDQVEA MTLADKIVVL
HisP    182  SALDPELVGE VLRI-MQQLA EE-GK--TMV V-VTHEMGFA RHVSSHVIFL
PstB    182  SALDPI-STG RIEE-LITEL KQ-DY--TVV I-VTHNMQQA ARCSDHTAFM
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P29     227  KNQEYHFYKS NKEI-N-SNI LDQVFKND
OppD    241  YAGRTMEYGK ARDV-F-YQP VHPYSIGL
MalK    208  DAGRVAQVGK PLAVPLSGRP FCRRIYRF
HisP    227  HQGKIEEEGD PEQV-F-GNP QSPRLQQF
PstB    226  YLGELIEFSN TDDL-F-TKP AKKQTEDY
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Fig. 3. Comparison of p29 with bacterial permease proteins. Protein sequences are aligned to demonstrate similarities between p29 and the peripheral membrane components of the bacterial transport systems for oligopeptides (oppD), maltose (malK), histidine (hisP) and phosphate (pstB). Sequences are from the sources indicated in the text. Gaps were introduced to optimize similarities. Above the alignment the similarity between p29 and oppD is shown. Identical residues and conservative substitutions are indicated by bars and double dots respectively. Similarities between all five sequences are visualized below the sequence alignment. Identical and conserved residues, appearing in all five sequences, are indicated by vertical arrow heads and double dots respectively. Positions with identical residues and conserved substitutions present in four of the five sequences are labelled by a single dot. Amino acid substitution scoring 0 and higher in the log odd matrix of 250 PAMs (Dayhoff, 1978) were assigned to be conserved.

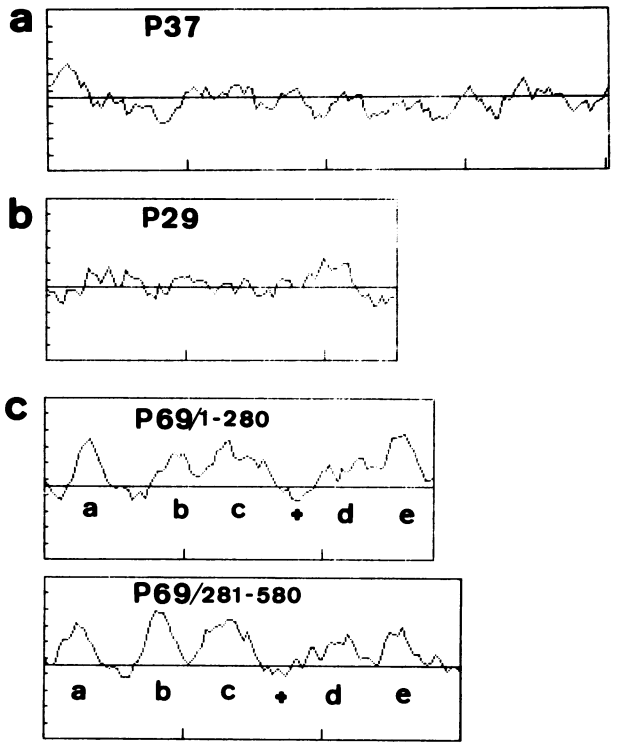


Fig. 4. Hydropathy plots of the polypeptides p37 (**a**), p29 (**b**) and p69 (**c**). The hydropathy profiles were obtained according to the method of Kyte and Doolittle (1982), using a window length of 19. Hydrophobic regions are above the line and hydrophilic regions below. In (c) the plots of the N-terminal half (residues 1−280) and the C-terminal half (residues 281−580) of p69 are arranged such that the profiles show clear congruency. The relatively hydrophilic region labelled (+) and the five potential membrane-spanning regions labelled a−e are assigned according to Hiles *et al.* (1987).

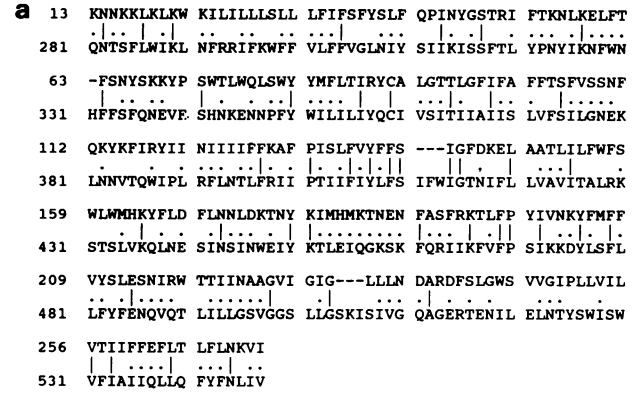
ORF 2 starts with nucleotide 1944 (Figure 2) and ends at position 2705. There is a potential ATG start codon at position 1950, although it is not preceded by a recognizable Shine−Dalgarno sequence. A hypothetical protein starting with the ATG would have 252 amino acids and a mol. wt of 29 kd, its six N-terminal amino acids overlapping the C terminus of p37. As outlined below, there is evidence that this protein, which we refer to as p29, is indeed expressed.

ORF 3 begins with nucleotide 2686 and ends at position 4440. In a situation analogous to ORF 2, there is a potential ATG initiation codon at position 2701, which is preceded by a potential Shine−Dalgarno sequence (Shine and Dalgarno, 1974). A hypothetical protein starting here would be 580 amino acids in length and have a mol. wt of 69 kd, its first two amino acids overlapping the C terminus of p29. We refer to it as p69.

Flanking this hypothetical operon are two sequences which have similarities to rho-independent transcription terminators (Rosenberg and Court, 1979). On the 5′ side of p37 there is a perfect 15-bp inverted repeat (arrows in Figure 2) with a row of seven uridinylates at its 3′ end. On the 3′ side of p69 there is a perfect 17-bp inverted repeat (arrows in Figure 2), allowing for two G−U base pairs, which is followed by four uridinylates. A promoter signal search gives the −10 and −35 consensus sequences underlined in Figure 2 as the most likely promoter region. This potential promoter region partly overlaps the upstream inverted repeat.

Hypothetical protein p29 is homologous to the ATP binding subunits of periplasmic binding-protein-dependent permeases of Gram-negative bacteria

We have compared the sequences of p37 and of the two hypothetical proteins p29 and p69 with the NBRF and EMBL protein sequence databases. No significant sequence similarities of p37 and p69 with other known proteins were found. However, extensive similarity of p29 with the bacterial proteins hisP of *Salmonella typhimurium* (Higgins *et al.*, 1982), malK of *E.coli* (Gilson *et al.*, 1982), oppD of *S.typhimurium* (Higgins *et al.*, 1985) and the more recently published pstB of *E.coli* (Surin *et al.*, 1985) was found (Figure 3). These fairly hydrophilic peripheral membrane proteins are part of the periplasmic binding-protein-dependent multicomponent transport systems for histidine, arginine, ornithine and lysine (his), for maltose and maltodextrins (mal), for phosphate (pst), and for oligopeptides (opp) of Gram-negative bacteria (reviewed in Ames, 1986). These proteins share extensive sequence similarities with each other and are thought to be energy coupling components because they all have nucleotide binding pockets, and because hisP, malK and oppD have been shown to bind



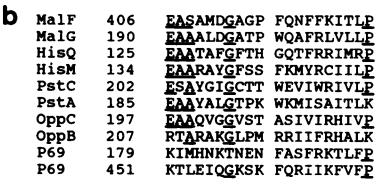


Fig. 5. Sequence similarities between the N-terminal and C-terminal halves of p69. (**a**) Sequence similarity between p69 (13−272) and p69 (281−547). Identical amino acids are indicated by vertical bars; dots indicate conserved residues. For the assignment of conserved substitutions see Figure 3. (**b**) Alignment of the conserved hydrophilic sequences of the integral membrane components of bacterial transport systems and the relatively hydrophilic stretches of p69, labelled (+) in Figure 4c. Conserved residues identified by Dassa and Hofnung (1985) are underlined.

ATP (Higgins *et al.*, 1985; Hobson *et al.*, 1984). There is evidence that these proteins interact both with the periplasmic substrate binding proteins and the hydrophobic integral membrane proteins which comprise the other components of the high-affinity transport systems (Ames and Spudich, 1976; Shuman and Silhavy, 1981; Higgins *et al.*, 1982; Ames and Higgins, 1983). p29 has 25% identical amino acid residues with oppD, and an additional 41% are conservatively changed. Similar numbers are obtained when p29 is compared with hisP, pstB and malK. Since the similarity of p29 with some of these proteins extends nearly to its N terminus (see Figure 3), we think it likely that p29 is indeed synthesized as shown in Figure 2. Figure 4B depicts the hydropathy plot of p29, showing that it is a fairly hydrophilic protein as are the ATP binding proteins of complex bacterial transport systems.

Hypothetical protein p69 has structural similarity to integral membrane proteins of bacterial periplasmic binding-protein-dependent transport systems

Analysis of the primary structure of p69 reveals that it consists of two similar parts. The alignment presented in Figure 5a shows that the N-terminal part (residues 13−280) shares 17% identical and an additional 32% conserved amino acids with the C-terminal part (residues 281−547). This internal duplication at the sequence level is also evident in the hydropathy plot. Figure 4c shows the nearly congruent hydropathy profiles of the N-terminal (residues 1−280) and the C-terminal (residues 281−580) parts of p69. The hydropathy profiles of the two halves are in turn very similar to the ones of the integral membrane components of bacterial transport systems (Hiles *et al.*, 1987). The four best characterized of these systems each have two very

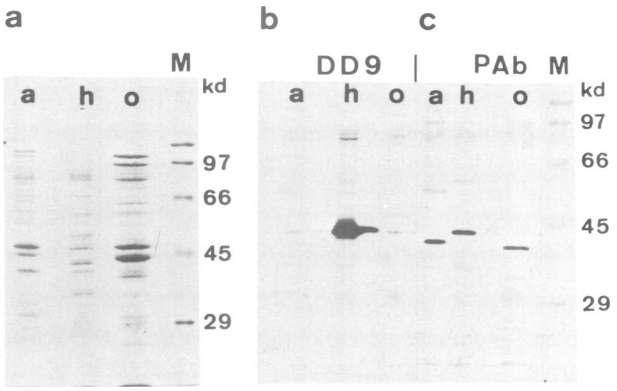


Fig. 6. SDS−polyacrylamide gel and Western blot analysis of total protein preparations from *M.arginini*, *M.hyorhinis* and *M.orale*. (**a**) Coomassie blue stained proteins from *M.arginini* (a), *M.hyorhinis* (h) and *M.orale* (o), separated on a 12% SDS−polyacrylamide gel. (**b** and **c**) Identical Western blots of proteins of the three mycoplasma species. Probing the transferred proteins with the monoclonal antibody DD9, only p37 from *M.hyorhinis* was detected (panel b, lane h). An affinity-purified rabbit antiserum directed against a synthetic N-terminal peptide of p37 (Pab), recognized individual proteins from *M.arginini* and *M.orale* as well as p37 in *M.hyorhinis* (panel c).

hydrophobic components (HisM, HisQ; OppB, OppC; PstA, PstC; MalF, MalG). All these components have very similar hydropathy profiles resembling closely the profiles of the two halves of p69. The hydrophilic regions labelled (+) in Figure 4c contain a sequence which has been shown to be conserved in the hydrophobic components of transport systems (Dassa and Hofnung, 1985). The alignment of the (+) regions of p69 with this conserved sequence of eight other hydrophobic transport proteins is shown in Figure 5b. Although no position is strictly conserved, some similarities can be recognized. We think that the internal duplication of p69, together with the close similarity of its hydropathy profiles to those of bacterial transport proteins presents evidence that p69 is indeed initiated and synthesized as shown in Figure 2.

p37 is a constituent of Mycoplasma hyorhinis

The experiments described above show that p37 is a prokaryotic protein, probably of mycoplasmal origin. Indeed, using DAPI (4′,6-diamidino-2-phenyl-indoldihydrochloride) DNA staining we found mycoplasma-like organisms tightly bound to the cell surface of many of our cell lines. To identify the species which p37 is associated with, we attempted to isolate and identify the mycoplasma species in our cell lines in collaboration with Dr R.H.Leach, Mycoplasma Reference Facility, NCTC, London. As described in detail elsewhere, three species could be identified. *M.arginini* was isolated from FS9 cells, *M.orale* from L929 cells and *M.hyorhinis* was identified in the p37-positive cell line MM96, but could not be cultured *in vitro*. Consequently we used *M.hyorhinis GDL*, a strain which grows *in vitro*. A genomic Southern analysis under low stringency conditions of the three mycoplasma species showed that only the DNA from *M.hyorhinis*, but not from *M.orale* and *M.arginini*, hybridized to our cloned probes (not shown).

Figure 6b shows a Western blot of the three mycoplasma species using the monoclonal antibody DD9. p37 is detected in *M.hyorhinis*, but not in the other two species. Furthermore, infection of L929 cells or mycoplasma-free FS9 cells with *M.hyorhinis*, but not with the other two species, resulted

in p37-positive cells (not shown). We conclude that p37 is a *M.hyorhinis* protein. This means that FS9 cells are contaminated with at least two mycoplasma species, namely *M.arginini* and *M.hyorhinis*, although the latter strain could not be isolated and cultivated *in vitro*. This has been confirmed by immunofluorescence staining of FS9 cells using antisera recognizing specifically individual mycoplasma species.

Figure 6c shows a Western blot identical to that in Figure 6b, except that it was probed with an affinity-purified rabbit antiserum directed against a synthetic peptide corresponding to amino acid 2–17 (SNTGVVKQEDVSVSQG) of the N-terminal sequence of p37. As can be seen, with *M.hyorhinis* a band identical in size to that obtained with monoclonal antibody DD9 is stained. In addition, however, this antiserum recognizes proteins in both *M.orale* and *M.arginini* that are slightly smaller in size than p37. This suggests that these two species have homologous proteins which have lost the epitope recognized by DD9. Their coding sequences have apparently diverged too far to be detected by cross-hybridization to the *M.hyorhinis* probes.

Discussion

The mycoplasma operon

Periplasmic binding-protein-dependent transport systems of Gram-negative bacteria consist of an ATP binding component, two very hydrophobic integral membrane proteins which are thought to form a complex with the ATP binding component, and specific receptor proteins located in the periplasm. With the exception of the ATP binding proteins, the components of different transport systems are not homologous. Nevertheless, some similarities do exist. The components of these transport complexes are coded for by one, or sometimes two, operons (reviewed in Ames, 1986). Furthermore, the periplasmic binding proteins are synthesized with a signal peptide, to allow their extracellular location. If we compare the hypothetical operon coding for p37, p29 and p69 with these bacterial transport complexes, the similarities are striking. Firstly, these three proteins appear to be coded by an operon, implying a functional connection between them. Secondly, p29 is homologous to the ATP binding components of such complexes to about the same degree as these are to each other. Thirdly, p69 is a very hydrophobic protein with the typical structure of the hydrophobic membrane proteins of transport systems. In the systems characterized most thoroughly two such components have been found. In some systems the two components have weak sequence similarities (e.g. 65 out of 306 identical amino acids in OppB and OppC; Hiles *et al.*, 1987). It has been proposed that these proteins arose by gene duplication and might function as a pseudodimer (Ames, 1986). The situation with p69 is very similar, except that the two components are expressed as two homologous domains of a single protein. Finally, p37 is synthesized with a signal peptide and is located on the outside of the cell membrane, although apparently tightly associated with it, possibly by modification of its N-terminal cysteine into a lipo amino acid (Weyer *et al.*, 1987; Wu, 1987). We hypothesize that p37 functions as a receptor of an unknown ligand.

In conclusion, our sequence data suggests that *M.hyorhinis* possesses a high-affinity transport systems similar to periplasmic binding-protein-dependent transport systems of Gram-negative bacteria. This result is rather unexpected as not only do mycoplasma lack a cell wall and periplasmic space but, as judged by their rRNA sequences (Woese *et al.*, 1980; Woese, 1987), are related to Gram-positive bacteria. However, biochemical evidence will be needed to confirm the function of the hypothetical operon and its products.

Effect of M.hyorhinis on contact inhibition of locomotion of mammalian cells

Contact inhibition of locomotion is disrupted by the presence of *M.hyorhinis* and can be restored with monoclonal antibodies against p37. Although we do not know the molecular mechanisms underlying this phenomenon, we have made a number of observations which must be taken into account.

Firstly, the mouse sarcoma cell line L929 used as a control in the confronted explant assays has a very low invasive index and does not contain p37 (Steinemann *et al.*, 1984a,b), but we found the cell line to be contaminated with *M.orale*, which contains a protein similar to p37. Elimination of *M.orale* from L929 cells does not alter the low invasivity index (C.Schmidhauser *et al.*, in preparation). However, infection of both mycoplasma-free and *M.orale*-contaminated L929 cells with *M.hyorhinis* greatly increases their invasivity, which in turn can be reduced to the background level by addition of DD9 Fab fragments. Thus, the effect of mycoplasma contamination on contact inhibition of locomotion is species specific, i.e. only *M.hyorhinis*, but not *M.orale*, enhances invasivity. This species specificity could be a consequence of the qualitative difference between p37 and its homologues in the other two species, or it could result from the fact that *M.hyorhinis* grows to much larger numbers on our cell lines than do *M.orale* and *M.arginini*, as we consistently observed.

Secondly, double staining of *M.hyorhinis*-contaminated FS9 cells with a DNA fluorescent dye and fluorescent p37-specific antibodies revealed that the mycoplasmas are located on the surface of the mammalian cells and that, within the limits of detection, p37 is exclusively associated with them. At present we do not know whether p37 or its function is directly involved in the influence of *M.hyorhinis* on contact inhibition. Blocking of p37 by specific antibody could alter the mycoplasma metabolism and thus indirectly modify their interaction with the host cells. Alternatively, p37 may be involved more directly. One could speculate that the permease which includes p37 changes the local concentration of its substrate(s). Such local changes may influence the mechanism of contact inhibition in the cellular host. Identification of the ligand(s) of p37 might help to clarify this point.

Thirdly, we have made two observations which may indicate the existence of a mammalian surface antigen which is recognized by the monoclonal antibody DD9 (C.Schmidhauser *et al.*, in preparation). Removal of mycoplasmas from FS9 cells significantly decreases their invasivity, but not to the ground level obtained in the presence of DD9. Furthermore, addition of DD9 to mycoplasma-free cells reduces their invasivity to the background level, whereas unspecific antibodies have no effect. Similar results were obtained with NIH 3T3 cells, which exhibit an intermediate level of invasiveness in the confronted explant assay: when infected with *M.hyorhinis*, their invasive behaviour is significantly enhanced. Addition of DD9

suppresses the invasivity of both infected and mycoplasma-free (i.e. p37 free) cells. These observations suggest that DD9 recognizes an epitope on mammalian cells similar to that on p37. Although we were unable to detect such an antigen on Western blots, by immunoaffinity chromatography or by immunofluorescent staining of fixed cells, we have indications from protein A binding assays that living tumour cells bind small amounts of DD9, whereas embryonic chicken heart fibroblasts do not. We are presently trying to confirm and identify this mammalian antigen. If it exists, it is not clear whether p37-specific Fab fragments are blocking two independent mechanisms of invasion.

Materials and methods

Purification of the p37 antigen

FS9 cells were cultured in Dulbecco-modified Eagle's medium (DMEM, Gibco) with 10% fetal calf serum (Inotech, Switzerland) at 37°C in 5% CO_2 in air (moist atmosphere). Crude membranes were prepared by solubilization of PBS (0.01 M Na,K-phosphate pH 7.2, 0.15 M NaCl) washed cells in 1% Triton X-100 and pelleting the nuclei at 10 000 *g* for 10 min. p37 antigen was purified from the extract by affinity chromatography. Anti-p37 specific mAb DD9 was coupled to Affi-Gel 10 (Bio-Rad) following the instructions of the manufacturer. The production and large-scale purification of mAb DD9 has been described elsewhere (Steinemann *et al.*, 1984b). The solubilized proteins from FS9 cells were incubated with the DD9-coated beads overnight at 4°C. The beads were washed six times in PBS, 0.5% Triton X-100 and elution was performed at room temperature with 4 M $MgCl_2$. The eluate from the antibody column was further purified by preparative SDS gel electrophoresis. Electroelution of p37 from the gels was performed exactly as described by Hunkapillar *et al.* (1983). The eluted protein fractions were concentrated by precipitation with 9 vol ethanol. Following this protocol, 10^9 FS9 cells yielded ~100 μg (2.5 pmol) of p37 antigen.

Cloning and sequencing of DNA encoding p37

For the determination of the N-terminal sequence of p37, an Applied Biosystems Inc. 470A gas-phase sequenator was used. PTH amino acids were analysed on a HPLC C18 column (200 × 2.1 mm) according to the instructions of the supplier (Applied Biosystems Inc.). Based on the determined N-terminal amino acid sequence, oligonucleotides of the sequence 5'AC(A,G)TC(T,C)TC(T,C)TG(T,C)TT(A,T,C,G)AC (degenerate positions are indicated in parentheses) were synthesized with an Applied Biosystems 380A synthesizer.

Cytoplasmatic DNA was isolated from a crude membrane fraction of FS9 cells (see above). To 2 ml of extract from ~4 × 10^8 cells, 1 vol of 0.2 M Tris–HCl, pH 7.5, 25 mM EDTA, 0.3 M NaCl, 2% (w/v) SDS and proteinase K to a final concentration of 200 μg/ml were added and incubated at 37°C for 30 min. After one phenol and one chloroform extraction, nucleic acids were ethanol precipitated and digested with 250 μg/ml RNase A in 50 mM Tris–HCl, pH 8.0, 10 mM EDTA at 37°C for 30 min. After extracting once with phenol and precipitating the DNA in 0.3 M sodium acetate with 0.55 vol propanol (10 min at room temperature), the sample was taken up in 40 μl TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). *Hin*dIII and *Xba*I restriction fragments of this cytoplasmatic DNA were used to construct corresponding libraries in pUC12. The libraries were screened with PAGE-purified, end-labelled oligonucleotides using standard procedures (Maniatis *et al.*, 1982). The sequence of the cloned DNA was determined by the chain termination method of Sanger *et al.* (1977), after subcloning the DNA into the bacteriophage M13 vector as described by Bankier and Barrell (1983).

In vitro growth of mycoplasma

M.arginini and *M.orale* were isolated from the cell cultures by Dr R.H.Leach, Mycoplasma Reference Facility, NCTC, London. They were grown at 37°C to log phase in media containing 1.8% (w/v) mycoplasma broth base (Oxoid), 0.7% (w/v) yeast extract (Oxoid), 20% horse serum, mycoplasma screened (Gibco), 0.025% (w/v) thallous acetate (Fluka Switzerland), sodium benzylpenicillin (Sigma) at 200 U/ml and 0.2% (w/v) L-arginine (Sigma). Phenol red was added to a final concentration of 0.0025% (w/v) in order to visualize growth of mycoplasma by a colour change from red to dark red. *M.hyorhinis GDL* was a gift from Dr R.H.Leach. They were grown under the same conditions, except that L-arginine was replaced by 0.1% glucose. Growth was monitored by the pH-dependent colour change from red to bright orange. Mycoplasmas were harvested by centrifugation at 10 000 *g* for 20 min and then washed three times in PBS.

Production of antipeptide antibody

Synthesis of the peptide. The N-terminal peptide NH_3-SNTGVVQED-VSVSQG(Y) (residues 2–17 of p37) with an additional tyrosine at its C-terminus (in parentheses) was synthesized in collaboration with B.Gutte (University of Zürich), using the solid-phase methods developed by Merrifield and his colleagues (for review see Marglin and Merrifield, 1970) with modifications introduced by Chang *et al.* (1980). After synthesis, the peptide was purified by gel filtration (Bio-Gel P-2, Bio-Rad, 98 × 2.5 cm column). Its purity was judged to be >90%, applying HPLC and amino acid analysis as criteria. The coupling of the peptide through the C-terminal tyrosine to bovine serum albumin (BSA) as carrier protein was accomplished by using *bis*-diazobenzidine as coupling reagent (Walter *et al.*, 1980). The reaction was monitored by the addition of *in vitro* labelled peptide (Jentoft and Dearborn, 1979) and the efficiency determined to be 4–9 mol. peptide/mol. BSA.

Immunization. New Zealand white rabbits (~6 months old) were injected s.c. with 750 μg protein in 1 ml PBS mixed with 1 ml of Freund's complete adjuvant. Booster injections, with 350 μg protein in Freund's incomplete adjuvant, followed 5 and 10 weeks later. The rabbits were bled 8 days after each booster injection. The blood was allowed to coagulate for 3–4 h at room temperature and the serum collected after centrifugation at 4°C for 15 min (1500 *g*).

Affinity purification. The affinity resin was prepared by coating Affi-Gel 10 beads (Bio-Rad) with synthetic peptide according to the instructions of the manufacturer. Antipeptide antibody was purified from whole serum by ammonium sulphate fractionation and by affinity chromatography following the method described for myosin light chain kinase antibodies (Guerriero *et al.*, 1981), except that 0.2% Triton X-100 in PBS was used as loading and washing buffer.

Western blotting

SDS–acrylamide gel electrophoresis (Laemmli, 1970) and Western blotting (Towbin *et al.*, 1979) were performed essentially as described by Steinemann *et al.* (1984b). The antibody-dilution and blot-washing buffer contained sodium PBS and 0.3% Tween-20. Affinity-purified antipeptide antibody was used at a concentration of 0.5 μg/ml. After incubation with a 1:600 dilution of peroxidase-conjugated goat anti-rabbit and rabbit anti-mouse immunoglobulin G (Nordic, Immunological Laboratories) respectively bound antibody was visualized by staining with 0.5 mg/ml 3',3-diaminobenzidine tetrahydrochloride (DAB) in PBS containing 0.015% H_2O_2.

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