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ZapA, the IgA-degrading metalloprotease of *Proteus mirabilis*, is a virulence factor expressed specifically in swarmer cells

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Summary

The IgA-degrading metalloprotease, ZapA, of the urinary tract pathogen *Proteus mirabilis* is co-ordinately expressed along with other proteins and virulence factors during swarmer cell differentiation. In this communication, we have used *zapA* to monitor IgA protease expression during the differentiation of vegetative swimmer cells to fully differentiated swarmer cells. Northern blot analysis of wild-type cells and β-galactosidase measurements using a *zapA*::*lacZ* fusion strain indicate that *zapA* is fully expressed only in differentiated swarmer cells. Moreover, the expression of *zapA* on nutrient agar medium is co-ordinately regulated in concert with the cycles of cellular differentiation, swarm migration and consolidation that produce the bull's-eye colonies typically associated with *P. mirabilis*. ZapA activity is not required for swarmer cell differentiation or swarming behaviour, as ZapA$^-$ strains produce wild-type colony patterns. ZapA$^-$ strains fail to degrade IgA and show decreased survival compared with the wild-type cells during infection in a mouse model of ascending urinary tract infection (UTI). These data underscore the importance of the *P. mirabilis* IgA-degrading metalloprotease in UTI. Analysis of the nucleotide sequences adjacent to *zapA* reveals four additional genes, *zapE*, *zapB*, *zapC* and *zapD*, which appear to possess functions required for ZapA activity and IgA proteolysis. Based on homology to other known proteins, these genes encode a second metalloprotease, ZapE, as well as a ZapA-specific ABC transporter system (ZapB, ZapC and ZapD). A model describing the function and interaction of each of these five proteins in the degradation of host IgA during UTI is presented.

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Introduction

Proteus mirabilis is a dimorphic bacteria that can undergo dramatic morphological and biochemical changes in response to growth on surfaces in a phenomenon known as swarmer cell differentiation and swarming behaviour. The subject of swarming and differentiation of *P. mirabilis* has been the subject of several recent reviews (Belas, 1996a,b; 1997), and the reader is referred to this literature for a more thorough discussion of the subject. In short, upon sensing either a solid surface or a viscous liquid environment, the vegetative swimmer cell responds by differentiating into an elongated, polyploid, highly flagellated swarmer cell (Belas, 1994). The co-ordinated interaction and communication of groups of swarmer cells is required for swarming motility and behaviour that ultimately result in the bull's-eye patterns observed on nutrient agar media.

Although *P. mirabilis* is often found in soil and water, it is also found in the human gastrointestinal tract, where it has access to the urinary tract. It is perhaps in this niche that it is most often encountered, as the causative agent of human urinary tract infections (UTIs). *P. mirabilis* is not commonly isolated as the cause of UTI in otherwise healthy individuals, but is associated with serious infections in patients with urinary tract abnormalities or long-term urinary catheterization (Senior, 1983). It is in these patients that infection with *P. mirabilis*, with its predilection for the tissues of the upper urinary tract (Fairley *et al*., 1971; Svanborg-Eden *et al*., 1980), can cause pyelonephritis and renal calculus (Senior, 1983).

The ability to invade cultured human urinary epithelial cells is associated with differentiated swarmer cells and not with undifferentiated swimmer cells (Allison *et al*., 1992a). To be able to colonize and invade urinary bladder epithelial cells, *P. mirabilis* produces several virulence factors (Allison *et al*., 1992b) whose expression is co-ordinated with swarmer cell differentiation (Allison *et al*., 1992a; 1994). Two of the major virulence factors that have been most thoroughly studied are urease (Mobley and Warren, 1987; Jones and Mobley, 1988; Johnson *et al*., 1993) and flagella (Belas, 1994; Gygi *et al*., 1995). *P. mirabilis*, however, also produces a host of other putative

or established virulence factors that include two haemolysins (Swihart and Welch, 1990a,b), four distinct types of fimbriae (Bahrani and Mobley, 1993; Massad and Mobley, 1994; Bijlsma *et al*., 1995; Cook *et al*., 1995; Massad *et al*., 1996), an amino acid deaminase (Massad *et al*., 1995) and ZapA, an IgA-degrading metalloprotease (Wassif *et al*., 1995).

IgA-degrading proteases have been studied in a number of bacterial pathogens, including *Neisseria meningitidis* and *N. gonorrhoeae* (Plaut *et al*., 1975), *Streptococcus pneumoniae* and *Haemophilus influenzae* (Kilian *et al*., 1979; Male, 1979). Interestingly, closely related, but non-pathogenic *Neisseria*, such as *N. lactamica* and *N. sicca*, do not produce IgA-degrading proteases (Kilian *et al*., 1983). The presence of IgA-degrading enzymes in pathogenic species, but their absence in related, but non-pathogenic species suggests a role for IgA protease activity in pathogenesis (Kilian *et al*., 1979).

ZapA is active against not only IgA1, but also IgA2 and IgG (Loomes *et al*., 1990; 1993). The gene encoding this enzyme, *zapA*, has been cloned and its sequence determined (Wassif *et al*., 1995). The recombinant 55 kDa IgA-degrading protease shows identical activity and specificity to the purified native *P. mirabilis* IgA protease; that is, it is active against both subclasses of IgA and IgG and is sensitive to EDTA (Wassif *et al*., 1995). The ZapA IgA protease is a member of the serralysin family of proteases, which include IgA- and IgG-specific proteases from *Serratia marcescens* and *Pseudomonas aeruginosa* (Doring *et al*., 1981; Molla *et al*., 1988), as well as the metalloproteases of *Erwinia chrysanthemi* (Letoffe *et al*., 1990).

In this report, we measured the expression of *zapA* during swarmer cell differentiation to correlate ZapA activity and pathogenesis. Our data indicate that ZapA is a virulence factor important in the maintenance or establishment of UTI and that it is expressed in swarmer cells, similar to other *P. mirabilis* virulence factors. Significantly, our analysis indicates that *zapA* is located within a locus of at least four other genes with similar functions, suggesting that *P. mirabilis* has developed a sophisticated mechanism to counter attacks made by host IgA and IgG defence systems.

Results

Cloning of zapA *and construction of a* zapA′::lacZ::′zapA *transcriptional fusion strain*

The *zapA* gene was cloned from an 8.6 kb fragment harboured on pCW101 (Table 1) using a polymerase chain reaction (PCR) with two oligonucleotide primers, Kpn3 and Kpn6. The resulting 1840 bp product contained the *zapA* ribosome binding site and terminator. This amplicon was cloned into pBluescript-II SK+ (Promega) using two genetically engineered *Kpn*I sites to produce pKW305 (Fig. 1A). To construct transcriptional fusions between *zapA* and *lacZ*, pKW305 was digested with *Bgl*II and ligated to the 4.5 kb *Bam*HI *lacZ*-Kan^R transcriptional fusion cassette from pLZK83 (Barcak *et al*., 1991) to generate pKW314. The *zapA*′::*lacZ*-Kan^R::′*zapA* fragment from pKW314 was then digested with *Kpn*I and ligated with *Kpn*I-digested pGP704 (Miller and Mekalanos, 1988) to generate pKW350 (Fig. 1A), which was used to transform *Escherichia coli* SM10 λ*pir*. Conjugation between *P. mirabilis* BB2000 and *E. coli* SM10 λ*pir* (pKW350) generated about 3000 *P. mirabilis* transconjugants that

Table 1. Strains, plasmids and oligonucleotides used.

Strain/plasmid/ oligonucleotide	Features	Source
E. coli DH5α λ pir	Nal^R	M. Donnenberg
E. coli SM10 λpir	Kan^R	Miller and Mekalanos (1988)
P. mirabilis BB2000	Rif^R	Belas *et al*. (1991)
P. mirabilis KW360	*zapA*::*lacZ*-Kan^R	This study
P. mirabilis KW362	*zapA*′::*lacZ*::′*zapA*-Kan^R merodiploid	This study
pLZK83	*lacZ*-Kan^R fusion vector	Barcak *et al*. (1991)
pGP704	Suicide vector, Amp^R	Miller and Mekalanos (1988)
pBluescript-II SK+	MCS, Amp^R	Promega
pCW101	8.6 kb *Eco*RI, *zapA*$^+$, *zapB*$^+$, *zapC*$^+$, *zapD*$^+$	Wassif *et al*. (1995)
pKW305	1.8 kb *zapA* in pBluescript-II SK+	This study
pKW314	pKW305 with *lacZ*-Kan^R	This study
pKW350	pGP704 with *zapA*::*lacZ*-Kan^R (from pKW314)	This study
Kpn3	5′-CTTATGGTACCCCCCTAG-3′	This study
Kpn6	5′-AATAGGTACCAATAGAGAG-3′	This study
ZapBF	5′-AGAGCGCTTCTTTAAAACCAACA-3′	This study
Zap32B	5′-ATTTATCTGGCGAATGCCATT-3′	This study

Amp^R, ampicillin resistance; Kan^R, kanamycin resistance; MCS, multicloning site; Nal^R, nalidixic acid resistance; Rif^R, rifampicin resistance.

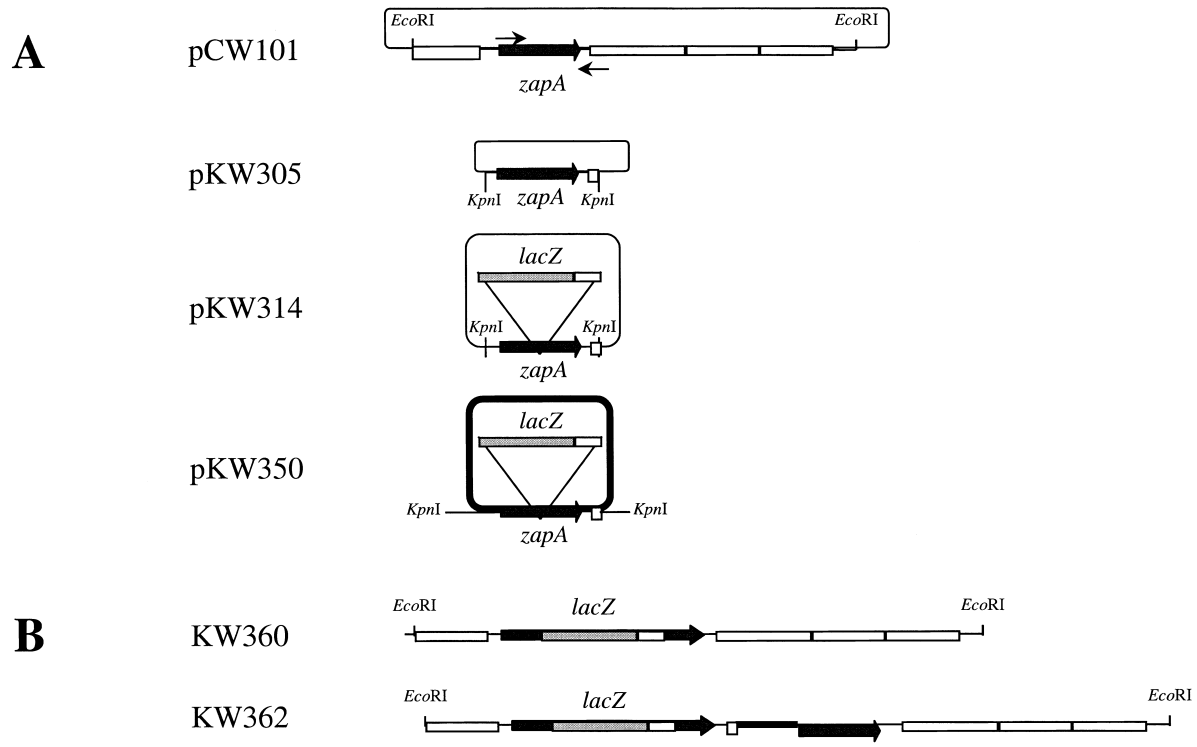

Fig. 1. Cloning and mutagenesis of *zapA*.
A. PCR primers Kpn3 and Kpn6 were used to produce a 1840 kb fragment that was cloned into pBluescript-II SK+ to generate pKW305. The 4.5 kb *Bam*HI *lacZ*-Kan^R from pLZK83 was cloned into the *Bgl*II site of pKW305, generating pKW314. The 6.34 *zapA*::*lacZ*-Kan^R *Kpn*I fragment from pKW314 was cloned into pGP704, generating the mutator plasmid pKW350.
B. Both double (KW360) and single cross-over (KW362) mutants were obtained from the conjugation of *P. mirabilis* BB2000 and *E. coli* SM10 λpir (pKW350).

were resistant to kanamycin, rifampicin and tetracycline. As shown in Fig. 1B, the results of this mating generated both a double cross-over knockout (KW360) and a single cross-over merodiploid strain (KW362).

The representative strains from both classes of mutant were analysed for their ability to produce the ZapA metalloprotease and degrade IgA. Three tests were used for this purpose, including the presence of a zone of clearing around ZapA$^+$ colonies growing on skimmed milk agar medium, as well as the ability of concentrated crude culture supernatants to degrade IgA (as detected by denaturing PAGE) and through spectrophotometric analysis using azocasein as a substrate. ZapA from wild-type *P. mirabilis* culture supernatants served as a positive control in these experiments and degraded IgA and azocasein (Table 2 and Fig. 2.).

Both classes of mutants produced from the allelic exchange mutagenesis were defective in ZapA activity.

Table 2. Phenotypes of ZapA mutants.

	Proteolysis			Swarming					
	Skimmed milk agar	Azocaseinase (%)	IgA	Flagella	Behaviour	Elongation	Motility	Urease	Haemolysis
P. mirabilis BB2000	+	100	+	+	+	+	+	+	+
P. mirabilis KW360	−	5.3	−	+	+	+	+	+	+
P. mirabilis KW362	−	2.0	−	NT	+	NT	+	+	+

Skimmed milk agar: a zone of clearing around a ZapA-producing colony; azocaseinase: the percentage of wild-type ZapA azopeptide-generating ability; IgA: the ability to degrade 4 μg of IgA *in vitro*; flagella: the presence of flagella when grown on LB agar, as viewed under a microscope; behaviour: the ability to produce the bull's-eye pattern of swarming on LB agar; elongation: the presence or absence of elongated swarmer cells, as viewed under a microscope; motility: the ability of bacterial cells to move from the site of inoculation in motility agar; urease: a buff-to pink colour change, indicating the degradation of urea; and haemolysis: the presence of a green discoloration surrounding colonies on TSA with 5% sheep red blood cells.

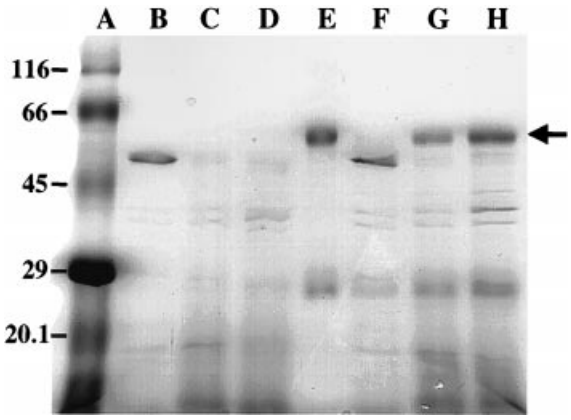

Fig. 2. The activity of *P. mirabilis* supernatants on IgA proteolysis. Concentrated crude culture supernatants from wild-type cells, double cross-over mutant KW360 and single cross-over strain KW362 are shown in lanes B, C and D respectively. Lanes F, G and H were loaded with the same supernatants plus 3 μg of IgA after an incubation for 13 h at 37°C. The arrow indicates the IgA band, and ZapA may be observed as the band of 55 kDa in lanes B and F. ZapA proteolysis of IgA can be observed in lane F (Zap^+), but not in lanes G or H containing supernatants from ZapA-deficient strains. Lane A contains protein size standards (in kDa), while an IgA control is shown in lane E.

Double cross-over strain KW360 and single cross-over strain KW362 show no zones of clearing in skimmed milk assays. $ZapA^-$ mutants produce less than 6% of the wild-type azocaseinase activity and are unable to degrade IgA, demonstrating a loss of ZapA activity (Table 2 and Fig. 2). To confirm that this mutation did not have pleiotropic effects, each strain was also tested for the production of two other virulence factors known to be induced during swarmer cell differentiation. Both KW360 and KW362 are equivalent to the wild type in their production of urease and haemolysin and demonstrated no defects in motility, swarmer cell elongation, flagellar production or swarmer cell behaviour (Table 2). These data indicate that *zapA* does not directly affect the expression of other virulence factors, and the mutation is specific to this gene.

The expression of zapA *is induced during swarmer cell differentiation and swarming behaviour*

The expression of *zapA* was measured using the *zapA′::lacZ::′zapA* fusion strain KW360 and by directly isolating and detecting *zapA* mRNA through Northern blot analysis. Using this transcriptional fusion, the β-galactosidase activity of swarmer cells removed from the periphery of an agar plate was compared with the activity of the enzymes in swimmer cells. The results, shown in Table 3, indicate that the expression of β-galactosidase increases 30-fold (1551 units versus 49 518 units) in the differentiated swarmer cells compared with swimmer cells in the double cross-over mutant. Similar results were also observed using the merodiploid strain (1045 units versus 34 634 units).

Table 3. β-Galactosidase activity (Miller units).

Strain	Swimmer (SEM)	Swarmer (SEM)
P. mirabilis BB2000	NA	NA
P. mirabilis KW360	1551 (4.64)	49518 (4025)
P. mirabilis KW362	1045 (4.94)	34634 (1518)

SEM, standard error of the mean.

Fig. 3. Cyclic regulation of *zapA* expression during swarmer cell differentiation and behaviour. *P. mirabilis* strain KW360 (*zapA′::lacZ::′zapA*) was inoculated onto the centre of L agar medium supplemented with the chromogenic indicator substrate Xgal. This medium permits swarmer cell differentiation and cyclic migratory behaviour and produces a bluish-green zone at areas of β-galactosidase activity. After 48 h of swarming behaviour, maximal expression of *zapA* (β-galactosidase activity) occurs just before the consolidation zone where both differentiation and motility cease.

β-Galactosidase activity of the *zapA′::lacZ::′zapA* fusion strain KW360 was measured as the cells underwent swarming migration behaviour on nutrient agar containing the chromogenic indicator Xgal. As can be observed in Fig. 3, KW360 produces a bull's-eye colony of concentric rings consistent with the colony patterns seen in wild-type *P. mirabilis*. ZapA expression, as determined by the blue chromogen Xgal, was consistent with the data obtained earlier. However, surprisingly, *zapA* is not expressed uniformly during swarming migration behaviour. Instead, as shown in Fig. 3, the level of β-galactosidase activity rises and falls during the swarmer cell migratory cycle. ZapA appears to be maximally expressed just before the consolidation phase, in which the cells dedifferentiate back to a swimmer cell morphology and stop active motility. These data suggest that *zapA* expression is tightly integrated with swarmer cell differentiation and with swarming behaviour.

To correlate the β-galactosidase measurements directly

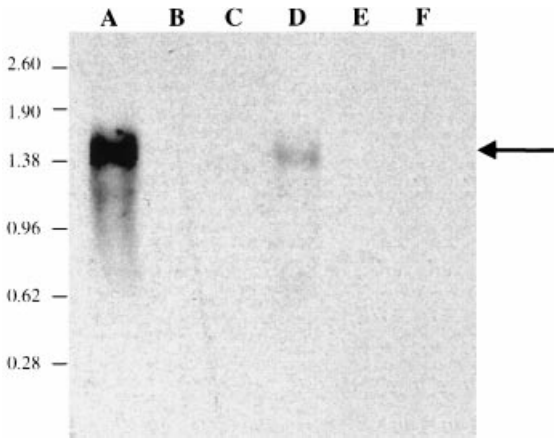

Fig. 4. Northern blot analysis of *zapA* mRNA. Total RNA was isolated from uninduced swimmer cells and fully differentiated swarmer cells. The lanes represent RNA samples from swarmer cells of wild type (A), KW360, a ZapA$^-$ strain (B) and the single cross-over KW362 (C), as well as RNA from swimmer cells of the same strains (lanes D–F respectively). A single band of hybridization is seen migrating at about 1.4 kb, corresponding to the *zapA* messenger RNA. Comparison of the density of the *zapA* transcript from swarmer cell and swimmer cells suggests that there is 20–30 times more *zapA* in the differentiated cells.

to *zapA* mRNA levels, Northern blots of total RNA isolated from wild-type swimmer and swarmer cells were prepared. When labelled probes specific to the *zapA* gene were hybridized to total cellular RNA separated by agarose gel electrophoresis, we observed a single band of 1.4 kb, corresponding to the *zapA* message. The intensity of this band differed dramatically when RNA from swimmer cells was compared with that extracted from swarmer cells (Fig. 4). The 1.4 kb transcript is not detected in either the double cross-over mutant (KW360) or the single cross-over strain (KW362).

Survival of P. mirabilis *during UTI is associated with ZapA*

As IgA-degrading proteases have long been considered putative virulence factors, we sought to confirm that the ZapA IgA-degrading metalloprotease did, in fact, impart a survival advantage to *P. mirabilis* during UTI. A mouse model of ascending UTI (Johnson *et al*., 1993) was chosen because of the unique ability of ZapA to degrade mouse IgA (Wassif *et al*., 1995), an unusual characteristic, as most IgA-degrading proteases are host IgA specific (Plaut, 1983; Proctor and Manning, 1990). Comparisons of CBA/JHSD mice infected by intraurethral catherization with wild-type *P. mirabilis* or ZapA-defective *P. mirabilis* show a decrease in survival when ZapA is mutated, indicating a decrease in the virulence of the ZapA$^-$ strain (Fig. 5 and Table 4). Figure 5 shows the mean of colony-forming units (cfu) ml^{-1} urine or g^{-1} bladder (or kidney) recovered from 10 mice infected with wild-type cells and nine mice infected with the ZapA-defective strain (KW360). Urine samples recovered from ZapA$^-$ *P. mirabilis*-infected mice contained only 1.90×10^2 cfu ml^{-1} compared with 1.67×10^7 cfu ml^{-1} from wild-type cells ($P=0.01$). Similar statistically significant differences exist for the bladder (1.85×10^1 cfu g^{-1} versus 3.07×10^5 cfu g^{-1}; $P=0.005$), left kidneys (3.42×10^2 cfu g^{-1} versus 5.01×10^4 cfu g^{-1}; $P=0.008$) and right kidneys (7.17×10^2 cfu g^{-1} versus 2.43×10^4 cfu g^{-1}; $P=0.008$). These results underscore the importance of ZapA in mouse UTI and represent the first experimental evidence proving that a bacterial IgA-degrading protease is a necessary virulence factor.

Sequence analysis of the zap *locus*

The observation of a single 1.4 kb *zapA* transcript was unexpected as, in previously published work (Wassif *et al*., 1995), we have shown that other nearby loci were necessary for ZapA expression, suggesting that *zapA* resided in an operon consisting of other genes essential for its expression. For this reason, we obtained the nucleotide sequence of a region of 9349 bp around and including the *zapA* gene. A total of five open reading frames (ORFs), each oriented in the same direction of transcription, are

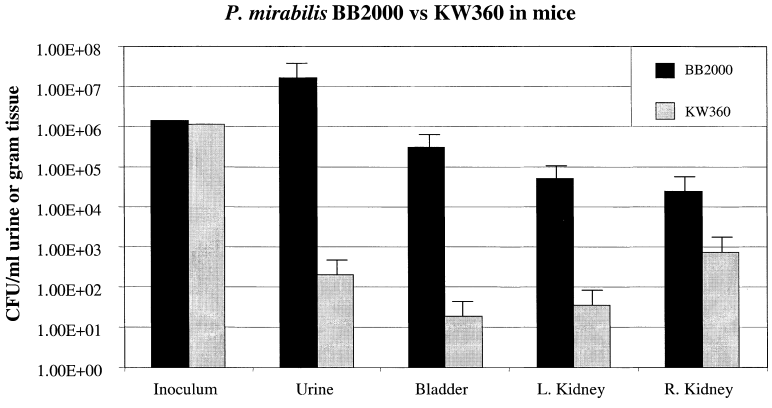

Fig. 5. Recovery of ZapA$^-$ *P. mirabilis* from the mouse urinary tract after UTI. A mouse model of ascending UTI was used to measure the change in survival and virulence of the ZapA-defective strain, comparing it with wild type. After infection, cells were recovered and cfu determinations were made from samples of urine, bladder and both left and right kidneys. Loss of ZapA adversely affects survival, as seen in comparisons of wild-type and ZapA$^-$ counts.

Table 4. Recovery of bacteria from mouse urinary tract sites.

Strain	Inoculum	Urine (SEM)	Bladder (SEM)	Left kidney (SEM)	Right kidney (SEM)
P. mirabilis BB2000	1.44×10^{6}	1.67×10^{7} ($\pm 2.12\times10^{1}$)	3.07×10^{5} ($\pm 8.61\times10^{0}$)	5.01×10^{4} ($\pm 7.66\times10^{0}$)	2.43×10^{4} ($\pm 6.46\times10^{0}$)
P. mirabilis KW360	1.12×10^{6}	1.90×10^{2} ($\pm 1.44\times10^{1}$)	1.85×10^{1} ($\pm 7.36\times10^{0}$)	3.42×10^{2} ($\pm 5.92\times10^{0}$)	7.17×10^{2} ($\pm 8.34\times10^{0}$)

contained within the *zap* locus. In order, from left to right, as represented in Fig. 6, these ORFs have been designated *zapE* (nucleotides 245–2308), *zapA* (nucleotides 2864–4339), *zapB* (nucleotides 4502–6241), *zapC* (nucleotides 6258–7583) and *zapD* (nucleotides 7583–8935). This sequence has been submitted to the EMBL/GenBank database and received accession number AF064762.

The 2064 bp ORF immediately preceding *zapA*, called *zapE*, encodes a protein of 687 amino acid residues and is an acidic protein (isoelectric point of pH 5.7). Computer-assisted searches of the amino acid sequence databases reveal significant homology between ZapE and other extracellular proteases, including PrtC (45% identity) and PrtA (38%) from *Erwinia chrysanthemi* (Ghigo and Wandersman, 1992), as well as a 44% identity to serralysin from *S. marcescens*, 36% identity to *P. aeruginosa* AprA (Duong *et al*., 1992) and 42% identity with ZapA (Wassif *et al*., 1995). In addition, between D-463 and D-545, ZapE contains four repeats of a glycine- and aspartic acid-rich 19-amino-acid motif [D-x-(L,I)-x-x-x-x-G-x-D-x-(L,I)-x-G-G-x-x-x-D] (Baumann *et al*., 1993) frequently associated with Ca^{2+}-dependent haemolysins and cytolysins (Felmlee *et al*., 1985; Lo *et al*., 1987; Kraig *et al*., 1990; Chang *et al*., 1991; Jansen *et al*., 1993). As is the case with ZapA, ZapE also contains a zinc-binding region between V-286 and L-295 that is common to many zinc-dependent metalloproteases (Baumann, 1994), suggesting that ZapE may function in a manner similar to ZapA.

The 556 bp intergenic region between *zapE* and *zapA* contains a very unusual feature. In addition to a potential rho-independent terminator for *zapE* (starting at nucleotide 2315) and a possible *zapA* promoter region at nucleotides 2728–2773, this intergenic region contains 11 copies, located at nucleotides 2457–2533, of the heptameric sequence CAAACAT in a direct tandem repeat 321 bases upstream of the start codon of *zapA* (nucleotide 2864). This places the direct repeat region immediately 5′ to the putative *zapA* promoter, suggesting that the direct repeat may serve a function in the transcriptional control of *zapA*, reminiscent of enhancer sequences such as the ToxR binding site of *Vibrio cholerae* (Miller *et al*., 1987).

The sequence and analysis of *zapA* has been published previously (Wassif *et al*., 1995) and will not be repeated

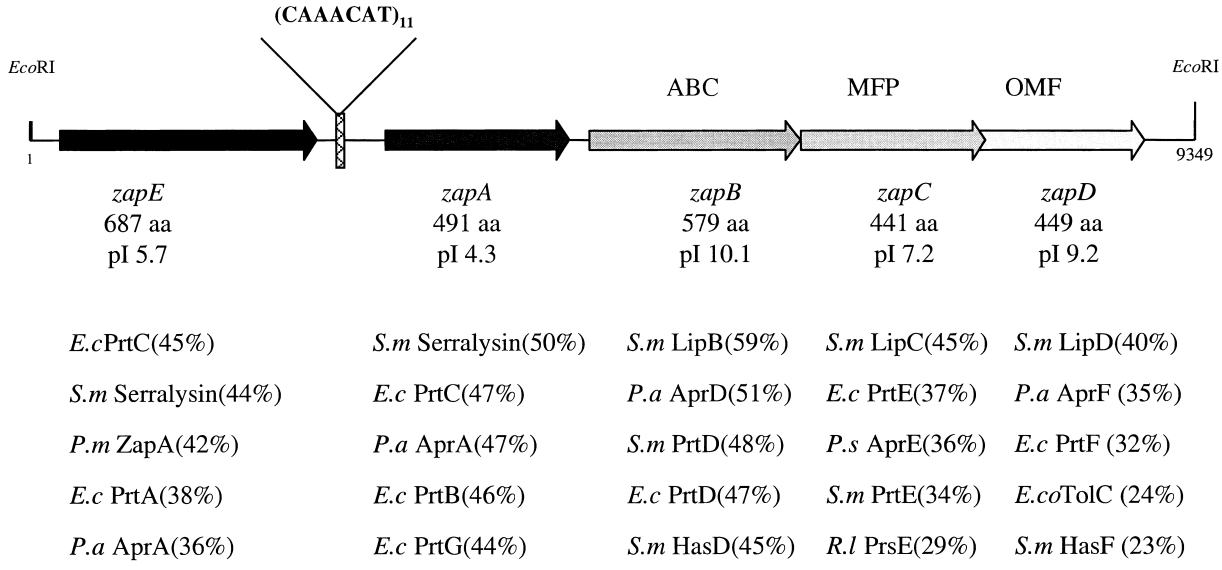

Fig. 6. Organization of the *zap* genetic locus. Contained within a 9349 bp region are five ORFs that are important for IgA degradation. Based upon their homology to known protein sequences, ZapE and ZapA are proteases, whereas ZapB, ZapC and ZapD form an ABC transporter system (ABC, ATP binding cassette; MFP, membrane fusion protein; and OMF, outer membrane factor). The five most homologous proteins to each of the ORFs are listed below the depiction of the sequence. Percentages refer to amino acid identity with deduced Zap locus proteins. *E.c*, *Erwinia chrysanthemi*; *S.m*, *Serratia marcescens*; *P.a*, *Pseudomonas aeruginosa*; *R.l*, *Rhizobium leguminosarum*; and *E.co*, *Escherichia coli*.

here. Following 3′ to the stop codon of *zapA* is an intergenic region of 163 bp containing the putative rho-independent terminator sequence of *zapA* (nucleotide 4345), a possible promoter (nucleotides 4384–4429) and ribosome binding site (nucleotide 4491) for the next three ORFs, *zapB*, *zapC* and *zapD*, which are likely to be transcribed as a polycistronic message based on the nucleotide sequence. The deduced amino acid sequence and corresponding homology to proteins in the databases suggests that ZapB, ZapC and ZapD encode proteins responsible for an ABC transport mechanism. Previous work has shown that mutations in this region adversely affect ZapA activity, implicating these gene products in the export of ZapA (Wassif *et al*., 1995).

ZapB is homologous to the ATP-binding cassette components of other transporter families (Fig. 6). ZapB also contains two important sequence features, an ATP/GTP binding site P-loop between G-365 and S-372 (Walker *et al*., 1982; Moller and Amons, 1985; Saraste *et al*., 1990; Koonin, 1993) and the ABC transporter motif present at L-468 and L-482 (Doolittle *et al*., 1986; Higgins *et al*., 1986). ZapC shares significant homology with the membrane-spanning fusion protein (MFP) components of the ABC transporter family (Finnie *et al*., 1998). Lastly, the deduced amino acid sequence of ZapD is similar to several outer membrane proteins associated with substrate transport, including LipD (40% identity) (Akatsuka *et al*., 1995) and HasF (23% identity) (Letoffe *et al*., 1994; Binet and Wandersman, 1996) from *S. marcescens*, AprF (35% identity) from *Pseudomonas aeruginosa* (Duong *et al*., 1992), PrtF (32%) from *E. chrysanthemi* (Delepelaire and Wandersman, 1991) and TolC from *E. coli* (Wandersman and Delepelaire, 1990; Letoffe *et al*., 1993). These results suggest that ZapA may be but one component in a much larger locus of genes with related function in IgA proteolysis and urinary tract pathogenesis.

Discussion

The *zapA* transcription data reveal an interesting pattern of regulation, suggesting that *zapA* expression is tightly co-ordinated not just with swarmer cell differentiation, but with swarming behaviour as well. In a broad sense, as is the case for other virulence factor genes, such as *ureC* (Allison *et al*., 1992a) and *flaA* (Belas, 1994), the transcription of *zapA* increases during the transition from swimmer to swarmer cells. On a more subtle level, however, swarming behaviour also modulates *zapA* transcription (Fig. 3). Careful analysis of the intensity of the β-galactosidase activity during the development of the swarming colony indicates that maximum *zapA* expression occurs at the boundary of the zone of consolidation. It is at this phase in the cycle of swarmer cell differentiation and migratory behaviour that differentiated swarmer cells dedifferentiated back into a swimmer cell morphology and when swarming motility and migration stop. Measurement of the cell density at different sections of the swarming colony indicates that the number of cells per unit area is roughly constant, discounting the possibility that the increase in β-galactosidase activity is the result of increased cell density at or near the consolidation zone. These experiments cannot distinguish whether *zapA* expression occurs as a consequence of this process of dedifferentiation or whether maximal ZapA activity induces swarming migration.

Strains defective in ZapA activity produce wild-type swarmer cells and swarming colonies, thus swarmer cell differentiation and migration behaviour do not require ZapA activity. These same ZapA$^-$ strains also produce wild-type levels of haemolysin and urease, indicating that there is no epistasis between *zapA* and either *ureC* or *hpmA*. This observation adds strength to the proposal that each of the virulence genes is under the control of a global swarmer cell regulator and their combined activities are required for the optimal survival of *P. mirabilis* during UTI.

In analysing the nucleotide sequence 5′ to the *zapA* start for clues to explain how the gene is regulated, we observed that the intergenic region contains 11 copies of the heptamer CAAACAT in a tandem direct repeat, which is similar to transcriptional control regions found near the promoter of other genes. The heptameric repeat region bears some similarity to regions associated with slipped strand mispairing, such as the CR sequence of *N. gonorrhoeae* (Stern *et al*., 1986). While we cannot rule out the possibility that a slipped strand mechanism is involved, no changes in the size of the region have been observed, based on PCR analysis, something that would be expected if such a mechanism of gene regulation were at work. Rather, we have preliminary data from Southwestern blots that the *zapA* heptameric repeat region binds a single protein expressed solely during swarmer cell differentiation (unpublished data). We are in the process of identifying this protein under the hypothesis that the binding of this protein to the heptamer repeat region may affect *zapA* expression.

Studies have shown that an IgA-degrading protease activity is present in the urine of patients with *P. mirabilis* UTI (Senior *et al*., 1991; Loomes *et al*., 1992), suggesting that ZapA participates in urinary tract pathogenesis. IgA-degrading proteases have been considered as virulence factors, because many mucosal pathogens produce them. While the correlation between IgA-degrading proteolysis and virulence suggests that these enzymes are virulence factors, this fact has not been proven *in situ*. The lack of proof is caused in part by a scarcity of animal models to examine the role of these proteases in disease, and this results from the host substrate specificity of many of the

IgA-degrading proteases. For example, the *Streptococcus pneumoniae* IgA-degrading protease cleaves only human, gorilla and rhesus monkey IgA, the natural hosts for *S. pneumoniae*, but the IgA of other mammalian species is unaffected (Proctor and Manning, 1990). In contrast, ZapA is unique among these enzymes, because its substrate specificity extends to both human and mouse IgA (Wassif *et al*., 1995). This unique characteristic permitted the evaluation of *P. mirabilis* strains defective in ZapA expression in a mouse model of ascending UTI.

The data from the mouse UTI experiments reveal that, in the absence of ZapA, bacterial survival in the urinary tract is dramatically attenuated. The rates of recovery of ZapA$^-$ *P. mirabilis* from the urine differ by at least $10^4\,ml^{-1}$, and from the bladder by $10^3\,g^{-1}$, compared with the recovery of wild-type *P. mirabilis* from those same sites. This represents the first demonstration of the significance of an IgA-degrading protease in UTI and provides the first evidence confirming the idea that these enzymes are virulence factors. These experiments also demonstrate that bacterial survival is influenced by the location of the infection within the urinary tract. For instance, in comparisons between the wild-type and a ZapA$^-$ strain, bacterial counts obtained from the kidneys were not as dramatically different as those from either urine or bladder. *P. mirabilis* favours long-term colonization in the upper urinary tract (Fairley *et al*., 1971; Svanborg-Eden *et al*., 1980), thus it is possible that kidney tissues produce less IgA or other host defence molecules. In this event, once bacteria have moved beyond the ureter and bladder into the kidney, cells defective in ZapA would have an equal chance of survival with wild-type bacteria.

We also analysed the nucleotide sequence on either side of *zapA*, because previous work (Wassif *et al*., 1995) had demonstrated that transposon insertions in nucleotide sequences downstream of *zapA* adversely affected proteolytic activity. Four additional ORFs were discovered that appear to have properties required for *zapA* function. ZapB, ZapC and ZapD share homology with the family of ABC transporter–extracellular protease exporter proteins (Higgins, 1992; Fath and Kolter, 1993) and probably function to export ZapA. This is in keeping with the analysis of ZapA (Wassif *et al*., 1995), which demonstrated that the C-terminal end of ZapA possessed an amino acid motif homologous to other ABC-transported proteins (Delepelaire and Wandersman, 1990; Duong *et al*., 1996). ZapA export is therefore different from the secretion mechanism of the *Neisseria* IgA protease, which involves Sec-mediated transport and an autotransporter encoded by the C-terminal portion of the *iga* gene (Pohlner *et al*., 1987). We are presently constructing *P. mirabilis* mutants in *zapB*, *zapC* and *zapD* to assess their function in the transport of ZapA and its role in *P. mirabilis* pathogenesis.

The ORF upstream of ZapA suggests that a second metalloprotease, ZapE, is encoded by the *zap* locus. ZapE shares homology with ZapA and other serralysin family proteases but, unlike ZapA, it lacks a C terminal signal sequence that is a hallmark of ABC-transported proteins (Baumann *et al*., 1993; Letoffe *et al*., 1994; Wassif *et al*., 1995). This casts doubt as to whether the Zap ABC transporter also exports ZapE, although it should also be noted that ZapE also lacks a defined N-terminal signal sequence. ZapE is probably an extracellular enzyme, as it shares a C-terminal Ca^{2+}-binding motif in common with other exported proteins. This motif is often seen as a single copy in most extracellular proteases and is present in as many as five copies in extracellular cytolysins and haemolysins (Baumann *et al*., 1993).

What is the function of the *zap* locus in the survival and adaptation of *P. mirabilis* during UTI? Many bacterial pathogens use extracellular enzymes, often exporting these proteins by means of ABC transporter systems. Our data provide evidence that the ZapA IgA-degrading metalloprotease is an essential virulence factor required for the survival of *P. mirabilis* during UTI. As noted earlier, this is the first evidence directly associating an IgA-degrading protease with enhanced survival during urinary tract pathogenesis. Equally important is the observation of the co-ordinate expression of *zapA* with swarmer cell differentiation and swarmer cell migratory behaviour. These data indicate that a finely tuned, biphasic mechanism controls ZapA activity during the colonization and invasion of the urinary epithelial cells. The role of ZapE in *P. mirabilis* UTI is currently not known. However, it is likely that it too is a protease, which perhaps acts in concert with ZapA to degrade host defence proteins or damage host tissues. If true, this suggests that *P. mirabilis* has a very capable mechanism for dealing with host defences during urinary tract pathogenesis.

Experimental procedures

Bacterial strains

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1. All bacterial cultures were incubated overnight at 37°C, unless otherwise noted. *P. mirabilis* strains were maintained on LSW$^-$ agar (10 g tryptone, 5 g yeast extract, 0.4 g NaCl, 50 ml glycerol, 20 g agar per litre dH_2O), while *E. coli* strains were maintained on LB agar (10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar per litre dH_2O). Antibiotics were added to the media at the following concentrations (per ml), unless otherwise noted: carbenicillin, 50 μg (for ampicillin resistance); rifampicin, 100 μg; kanamycin, 10 μg; and tetracycline, 6 μg.

Urease

A buff-to-pink colour change was used to detect urease

activity on urea agar (Difco) plates inoculated with *P. mirabilis* colonies incubated for 6–8 h at 37°C.

Haemolysin

Haemolytic activity was detected as a zone of greenish discoloration surrounding *P. mirabilis* colonies grown on trypticase soy agar (TSA) with 5% sheep red blood cells (TSA II; Becton Dickinson Microbiology Systems) after an overnight incubation at 37°C.

Motility

Motility was assessed on plates composed of motility agar (10 g tryptone, 5 g NaCl, 3.5 g agar per litre dH_2O) after overnight incubation at 37°C. A zone of migration radiating from the site of inoculation was used to indicate motility.

Swarmer cell assays

Aliquots (5 μl) of overnight LB broth cultures of strains to be tested for swarming were inoculated in the centre of 100 × 15 mm LB agar Petri dishes, incubated at 30°C for 16 h, then examined for the bull's-eye ring formation typical of this species. Cells obtained from the swarming colony periphery were examined by light microscopy for the presence of elongated, polyploid swarmer cells, motility and flagella.

Protease activity

Three assays were used to detect and measure protease activity. First, strains to be tested for ZapA protease activity were streaked on skimmed milk agar consisting of 15 g proteose peptone (Difco Laboratories), 10 g skimmed milk powder (Carnation) and 15 g agar per litre dH_2O, and incubated overnight at 37°C. A positive reaction consisted of a zone of clearing of the agar surrounding the protease-positive colony.

Secondly, enzymatic release of azo dye from azocasein was used to measure protease activity in a modified assay (Loomes *et al*., 1990). An azocasein solution consisting of 25 mg of azocasein dissolved in 1 ml of 2 mM $CaCl_2$, 50 mM Tris (pH 8.0) was prepared and sterilized by filtration. Overnight cultures (5 ml) of *P. mirabilis* were centrifuged and resuspended in cold 1× PBS (0.139 M NaCl, 0.002 M KCl, 0.008 M Na_2HPO_4, 1 mM KH_2PO_4) to an OD_{600} of 0.1 units. Ten OD_{600} units of bacteria were added in 100 μl of 2 mM $CaCl_2$, 50 mM Tris (pH 8.0) to 900 μl of azocasein solution and incubated for 16 h at 37°C. The reaction was terminated by the addition of 250 μl of 5% trichloroacetic acid. Centrifugation at 17 500 × *g* was used to remove unhydrolysed azocasein, and the resulting supernatants were added to 375 μl of 0.5 M NaOH. The amount of hydrolysed azocasein was determined from the A_{440}. Numbers reported are percentage azocaseinase activity of wild-type cells.

The final assay measured IgA degradation. In this test, 2 ml aliquots of 16 h *P. mirabilis* LB broth cultures were centrifuged for 15 min at 17 500 × *g* to pellet cells. The culture supernatants were applied to Centricon-3 concentrators (Amicon) and centrifuged for 90 min at 7500 × *g*. Aliquots (3 μg) of human IgA from plasma (Calbiochem-Novabiochem) were added to 40 μl aliquots of culture supernatants from *P. mirabilis*. After 13 h incubation at 37°C, the supernatant–IgA mixtures were subjected to 12.5% SDS–PAGE (Laemmli, 1970).

Conjugation

Filter conjugations were performed according to the method of Belas *et al*. (1991). Briefly, 1 ml cultures of LB broth supplemented with antibiotics were inoculated with single colonies of *P. mirabilis* B2000 or *E. coli* SM10 λ*pir* (pKW350) and incubated for 16 h at 30°C. Aliquots (200 μl) were harvested from each and centrifuged for 5 min at 8950 × *g*, washed in 1× PBS (0.139 M NaCl, 0.002 M KCl, 0.008 M Na_2HPO_4, 1 mM KH_2PO_4), resuspended in 50 μl of LB broth and applied to sterile 0.22 mm pore-sized filters (MSI) on LB agar (Sambrook *et al*., 1989). After 16 h incubation at 30°C, cells were harvested from the filters in 5 ml of 1× PBS, and 100 μl aliquots were plated on LSW^- agar with rifampicin, tetracycline and kanamycin.

β-Galactosidase activity

P. mirabilis metalloprotease mutants were grown overnight at 30°C in A broth (0.006 M K_2HPO_4, 0.03 M KH_2PO_4 $(NH_4)SO_4$, 0.002 M sodium citrate) supplemented with 10 g l^{-1} tryptone. Agar (15 g l^{-1} A broth) was used to produce A agar medium. Aliquots (0.5 ml) from the broth cultures were harvested and solubilized with $CHCl_3$ and 0.1% SDS. These solubilized aliquots were mixed with 4.5 ml of Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol) and 1 ml of 4 mg ml^{-1} ONPG, and the adsorbance at 420 nm, 550 nm and 600 nm was measured, as described by Miller (1972). Cells were also harvested from A agar plates in 3 ml of ice-cold 1× PBS and treated as above. The values reported are the means of OD readings from duplicate samples run in triplicate. Miller units are derived using the formula: $1000 \times (A_{420} - (1.75 \times A_{550})/(\text{time} \times 0.1 \times A_{600})$.

Expression of β-galactosidase activity during swarming behaviour in *P. mirabilis* metalloprotease mutants was monitored on plates in the following modified swarmer cell assay. Aliquots (10 μl) of overnight broth cultures were inoculated in the centre of 150 × 15 mm Petri plates containing LB agar with 96 μg ml^{-1} Xgal and incubated at 20°C for 48 h.

Analysis of ZapA⁻ strains during UTI

The ability of *P. mirabilis* to be recovered from various sites in the urinary tract was assessed using the CBA/JHSD mouse model of ascending UTI according to the method of Johnson *et al*. (1987). Two sets of 10 mice were infected with either 1.44×10^6 *P. mirabilis* BB2000 or 1.12×10^6 *P. mirabilis* KW360. The mice were sacrificed after 7 days, and bacterial colony counts (cfu) were determined from the urine, urinary bladder and the left and right kidneys of each mouse. The values reported are the means of cfu ml^{-1} urine or g^{-1} bladder or kidney. Results from mice challenged with wild-type *P. mirabilis* or *P. mirabilis* KW360 were compared by Student's *t*-test using the PC-PITMAN suite of programs (Dallal, 1986).

Recombinant DNA methods

Recombinant DNA techniques were performed according to standard protocols (Ausubel *et al*., 1989; Sambrook *et al*., 1989). Ligations were performed in low-melting-point agarose (Fisher Biotech) according to the following protocol. DNA fragments were separated by gel electrophoresis and excised from 0.8% TAE low-melting-point agarose using long-wave UV illumination and melted at 65°C for 10 min. Aliquots (5 μl) of both insert and vector were mixed together and placed at 65°C for an additional 5 min, then placed at 37°C for 10 min. A ligase–buffer mixture (1 μl of 10× ligase buffer, 1 μl of T4 DNA ligase and 9 μl of dH_2O) was added to the DNA in agarose and incubated at 20°C for 16 h. Before transformation, the mixture was heated to 65°C and diluted 1:5 with dH_2O.

Total cellular RNA was prepared from wild-type and KW360 swimmer and swarmer cells according to the method of Ausubel *et al*. (1989). Swimmer cells were obtained by inoculating 10 μl aliquots of overnight LB broth cultures into 2 ml of fresh LB broth and incubating at 37°C for 6 h with vigorous aeration. A 10 μl aliquot of an overnight LB broth culture was inoculated onto an LB agar plate and incubated for 6 h at 37°C to permit the differentiation into swarmer cells. Total cellular RNA (10 μg) was separated by electrophoresis on a 1.2% agarose in 1× MOPS buffer (0.2 M MOPS, 0.05 M sodium acetate, 0.05 mM EDTA) gel, transferred to nylon membranes (MSI) and hybridized to the 1.84 kb *Kpn*I *zapA* clone. Transcripts homologous to the 1.84 kb *Kpn*I fragment were detected using the DIG High Prime labelling and detection system (Boehringer Mannheim).

Oligonucleotide primers were generated using an oligonucleotide synthesizer (Oligo 1000; Beckman Instruments).

Sequencing and analysis of the zap *region*

The *zap* region was sequenced with primers ZapBF and Zap32B using the dideoxy chain termination method (Sanger *et al*., 1977), the Prism ready reaction dye deoxy termination kit (Applied Biosystems) and *Taq* polymerase on a model 373A DNA sequencer (Applied Biosystems). The Genetic Computer Group suite of gene and protein analysis programs (Devereux *et al*., 1984) and the BLAST series of programs (Altschul *et al*., 1990; 1997; Gish and States, 1993) were used to analyse the 9349 kb *Eco*RI segment of DNA comprising *zapE* to *zapD*. The complete nucleotide sequence of this region has been submitted to the EMBL/GenBank database (accession number AF064762).

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References

Akatsuka, H., Kawai, E., Omori, K., and Shibatani, T. (1995) The three genes *lipB*, *lipC* and *lipD* involved in the extracellular secretion of the *Serratia marcescens* lipase which lacks an N-terminal signal peptide. *J Bacteriol* **177:** 6381–6989.

Allison, C., Lai, H., and Hughes, C. (1992a) Co-ordinate expression of virulence genes during swarm cell differentiation and population migration of *Proteus mirabilis*. *Mol Microbiol* **6:** 1583–1591.

Allison, C., Coleman, N., and Hughes, C. (1992b) Ability of *Proteus mirabilis* to invade human urothelial cells is coupled to mobility and swarming differentiation. *Infect Immun* **60:** 4740–4746.

Allison, C., Emody, L., Coleman, N., and Hughes, C. (1994) The role of swarm cell differentiation and multicellular migration in the uropathogenicity of *Proteus mirabilis*. *J Infect Dis* **169:** 1155–1158.

Altschul, S., Gish, W., Miller, W., Myers, E., and Lipman, D. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Altschul, S., Madden, T., Schaffer, A., Zhang, J., Zhang, Z., Miller, W., *et al*. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389–3402.

Ausubel, F., Brent, R., Kingston, R., More, D., Seidman, J., Smith, J., *et al*. (1989) *Short Protocols in Molecular Biology. A Compendium of Methods from Current Protocols in Molecular Biology*. New York: John Wiley Sons.

Bahrani, F., and Mobley, H. (1993) *Proteus mirabilis* MR/P fimbriae: molecular cloning, expression, and nucleotide sequence of the major fimbrial subunit gene. *J Bacteriol* **175:** 457–464.

Barcak, G., Chandler, M., Redfield, R., and Tomb, J.-F. (1991) Genetic systems in *Haemophilus influenzae*. *Methods Immun* **204:** 321–324.

Baumann, U. (1994) Crystal structure of the 50 kDa metalloprotease from *Serratia marcescens*. *J Mol Biol* **242:** 244–251.

Baumann, U., Wu, S., Flaherty, K., and McKay, D. (1993) Three dimensional structure of alkaline protease of *Pseudomonas aeruginosa*: a two domain protein with a calcium binding parallel beta roll motif. *EMBO J* **12:** 3357–3364.

Belas, R. (1994) Expression of multiple flagellin-encoded genes of *Proteus mirabilis*. *J Bacteriol* **176:** 7169–7181.

Belas, R. (1996a) *Proteus mirabilis* swarmer cell differentiation and urinary tract infection. In *Urinary Tract Infections: Molecular Pathogenesis and Clinical Management*. Mobley, H., and Warren, J. (eds). Washington, DC: American Society for Microbiology Press, pp. 271–298.

Belas, R. (1996b) Sensing, response and adaptation to surfaces: swarmer cell differentiation and behavior. In *Molecular and Ecological Diversity of Bacterial Adhesion*. Fletcher, M. (ed.). New York: Wiley-Liss, pp. 281–331.

Belas, R. (1997) *Proteus mirabilis* and other swarming bacteria. In *Bacteria as Multicellular Organisms*. Shapiro, J., and Dworkin, M. (eds). New York: Oxford University Press, pp. 183–219.

Belas, R., Erskine, D., and Flaherty, D. (1991) Transposon mutagenesis in *Proteus mirabilis*. *J Bacteriol* **173:** 6289–6293.

Bijlsma, I., van Dijk, L., Kusters, J., and Gaastra, W. (1995) Nucleotide sequences of two fimbrial subunit genes,

pmpA and *ucaA* from canine-uropathic *Proteus mirabilis* strains. *Microbiology* **141:** 1349–1357.

Binet, R., and Wandersman, C. (1996) Cloning of the *Serratia marcescens hasF* gene encoding the Has ABC exporter outer membrane component: a TolC analogue. *Mol Microbiol* **22:** 265–273.

Chang, Y.-F., Young, R., and Struck, D. (1991) The *Actinobacillus pleuropneumoniae* hemolysin determinant: unlinked *appCA* and *appBD* loci flanked by pseudogenes. *J Bacteriol* **173:** 5151–5158.

Cook, S., Mody, N., Valle, J., and Hull, R. (1995) Molecular cloning of *Proteus mirabilis* uroepithelial cell adherence (*uca*) genes. *Infect Immun* **63:** 2082–2086.

Dallal, G. (1986) *STAT-SAK Computer Programs.* Malden, MA: STAT-SAK.

Delepelaire, P., and Wandersman, C. (1990) Protein secretion in Gram-negative bacteria: the extracellular metalloprotease B from *Erwinia chrysanthemi* contains a C-terminal secretion signal analogous to that *of Escherichia coli* alpha-hemolysin. *J Biol Chem* **265:** 17118–17125.

Delepelaire, P., and Wandersman, C. (1991) Characterization, localization and transmembrane organization of the three proteins PrtD, PrtE, and PrtF necessary for protease secretion by the Gram-negative bacterium *Erwinia chrysanthemi*. *Mol Microbiol* **5:** 2427–2434.

Devereux, J., Haeberli, P., and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12:** 387–395.

Doolittle, R., Johnson, M., Husain, I., van Houten, B., Thomas, D., and Sancar, A. (1986) Domainal evolution of a procaryotic DNA repair protein and its relationship to active transport proteins. *Nature* **323:** 451–453.

Doring, G., Orbernesser, H.-J., and Botzenhart, K. (1981) Extracellular toxins of *Pseudomonas aeruginosa*. Effect of two proteases on human immunoglobulins IgG, IgA and secretory IgA. *Zentrabl Bakteriol Parasitenkd Infektionskr Hyg Abt I Org Reihe A* **249:** 89–98.

Duong, F., Lazdunski, A., Cami, B., and Murgier, M. (1992) Sequence of a cluster of genes controlling synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretory pathways. *Gene* **121:** 47–54.

Duong, F., Lazdunski, A., and Murgier, M. (1996) Protein secretion by heterologous bacterial ABC-transporters: the C-terminus secretion signal of the secreted protein confers high recognition specificity. *Mol Microbiol* **21:** 459–470.

Fairley, K., Carson, N., Gutch, R., Leighton, P., Grounds, A., Laird, E., *et al.* (1971) Site of infection in acute urinary tract infections in general practice. *Lancet* **2:** 615–618.

Fath, M., and Kolter, R. (1993) ABC transporters: bacterial exporters. *Microbial Rev* **57:** 995–1017.

Felmlee, T., Pellett, S., and Welch, R. (1985) Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *J Bacteriol* **163:** 94–105.

Finnie, C., Zorreguieta, A., Hartley, N., and Downie, J. (1998) Characterization of *Rhizobium leguminosarum* exopolysaccharide glycanases that are secreted via type I exporter and have a novel heptapeptide repeated motif. *J Bacteriol* **180:** 1691–1699.

Ghigo, J.M., and Wandersman, C. (1992) Cloning, nucleotide sequence and characterization of the gene encoding the *Erwinia chrysanthemi* B374 PrtA metalloprotease: a third metalloprotease secreted via a C terminal secretion signal. *Mol Gen Genet* **236:** 135–144.

Gish, W., and States, D. (1993) Identification of protein coding regions by database similarity search. *Nature Genet* **3:** 266–272.

Gygi, D., Bailey, M., Allison, C., and Hughes, C. (1995) Requirement for FlhA in flagella assembly and swarm-cell differentiation by *Proteus mirabilis*. *Mol Microbiol* **15:** 761–769.

Higgins, C. (1992) ABC transporters from microorganisms to man. *Annu Rev Cell Biol* **8:** 67–113.

Higgins, C., Hiles, I., Salmond, G., Gill, D., Downie, J., Evans, I., *et al.* (1986) A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. *Nature* **323:** 448–450.

Jansen, R., Briaire, J., Kamp, E., Gielkens, A., and Smits, M. (1993) Cloning and characterization of the *Actinobacillus pleuropneumoniae*-RTX-Toxin III (ApxIII) gene. *Infect Immun* **61:** 947–954.

Johnson, D., Lockatell, C., Hall-Craigs, M., Mobley, H., and Warren, J. (1987) Uropathogenicity in rats and mice of *Providencia stuartii* from long-term catheterized patients. *J Urol* **138:** 632–635.

Johnson, D., Russell, R., Lockatell, C., Zulty, J., Warren, J., and Mobley, H. (1993) Contribution of *Proteus mirabilis* urease to persistence, urolithiasis, and acute pyelonephritis in a mouse model of ascending urinary tract infection. *Infect Immun* **61:** 2748–2754.

Jones, B., and Mobley, H. (1988) *Proteus mirabilis* urease: genetic organization, regulation, and expression of structural genes. *J Bacteriol* **170:** 3342–3349.

Kilian, M., Mestecky, J., and Schrohenloher, R. (1979) Pathogenic species of the genus *Haemophilus* and *Streptococcus pneumoniae* produce immunoglobulin A1 protease. *Infect Immun* **26:** 143–149.

Kilian, M., Thomsen, B., Petersen, T., and Bleeg, H. (1983) Occurrence and nature of bacterial IgA proteases. *Ann NY Acad Sci* **409:** 612–614.

Koonin, E. (1993) A super family of ATPases with diverse functions containing either classical or deviant ATP-binding motif. *J Mol Biol* **229:** 1165–1174.

Kraig, E., Dailey, T., and Kolodrubetz, D. (1990) Nucleotide sequence of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*: homology to the alpha-hemolysin/leukotoxin gene family. *Infect Immun* **58:** 920–929.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Letoffe, S., Delepelaire, P., and Wandersman, C. (1990) Protease secretion by *Erwinia chrysanthemi*: the specific secretion functions are analogous to those of *Escherichia coli* alpha haemolysin. *EMBO J* **9:** 1375–1382.

Letoffe, S., Ghigo, J.M., and Wandersman, C. (1993) Identification of two components of the *Serratia marcescens* metalloprotease transporter: protease SM secretion in *Escherichia coli* is TolC dependent. *J Bacteriol* **175:** 7321–7328.

Letoffe, S., Ghigo, J., and Wandersman, C. (1994) Secretion of the *Serratia marcescens* HasA protein by an ABC transporter. *J Bacteriol* **176:** 5372–5377.

Lo, R., Strathdee, C., and Shewen, P. (1987) Nucleotide sequence of the leukotoxin genes of *Pasteurella haemolytica* A1. *Infect Immun* **60:** 2276–2273.

Loomes, L., Senior, B., and Kerr, M. (1990) A proteolytic enzyme secreted by *Proteus mirabilis* degrades immunoglobulins of the immunoglobulin A1 (IgA1), IgA2 and IgG isotypes. *Infect Immun* **58:** 1979–1985.

Loomes, L., Senior, B., and Kerr, M. (1992) Proteinases of *Proteus* spp. purification, properties and detection in the urine of infected patients. *Infect Immun* **60:** 2267–2273.

Loomes, L., Kerr, M., and Senior, B. (1993) The cleavage of immunoglobulin G *in vitro* and *in vivo* by a proteinase secreted by the urinary tract pathogen *Proteus mirabilis*. *J Med Microbiol* **39:** 225–232.

Male, C. (1979) Immunoglobulin A1 protease production by *Haemophilus influenzae* and *Streptococcus pneumoniae*. *Infect Immun* **26:** 254–261.

Massad, G., and Mobley, H. (1994) Genetic organization and complete nucleotide sequence of the *Proteus mirabilis* PMF fimbrial operon. *Gene* **150:** 101–104.

Massad, G., Zhao, H., and Mobley, H. (1995) *Proteus mirabilis* amino acid deaminase: cloning, nucleotide sequence, and characterization of *aad*. *J Bacteriol* **175:** 5878–5883.

Massad, G., Fulkerson, J., Watson, D., and Mobley, H. (1996) *Proteus mirabilis* ambient-temperature fimbriae: cloning and nucleotide sequence of the *atf* gene cluster. *Infect Immun* **64:** 4390–44395.

Miller, J. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Miller, V., and Mekalanos, J. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **170:** 2575–2583.

Miller, V., Taylor, R., and Mekalanos, J. (1987) Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. *Cell* **48:** 271–279.

Mobley, H., and Warren, J. (1987) Urease-positive bacteriuria and obstruction of long-term catheters. *J Clin Microbiol* **25:** 2216–2217.

Molla, A., Kagimoto, T., and Maeda, H. (1988) Cleavage of immunoglobulin G (IgG) and IgA around the hinge region by protease from *Serratia marcescens*. *Infect Immun* **56:** 916–920.

Moller, W., and Amons, R. (1985) Phosphate-binding sequences in nucleotide-binding proteins. *FEBS Lett* **186:** 1–7.

Plaut, A.G. (1983) The IgA1 proteases of pathogenic bacteria. *Annu Rev Microbiol* **37:** 603–622.

Plaut, A., Gilbert, J., Artenstein, M., and Capra, J. (1975) *Neisseria gonorrhoeae* and *Neisseria meningitidis*: extracellular enzyme cleaves human immunoglobulin A. *Science* **190:** 1103–1105.

Pohlner, J., Halter, R., Beyreuther, K., and Meyer, T. (1987) Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. *Nature* **325:** 458–462.

Proctor, M., and Manning, P. (1990) Production of immunoglobulin A protease by *Streptococcus pneumoniae* from animals. *Infect Immun* **58:** 2733–2737.

Sambrook, J., Fritsch, F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74:** 5463–5467.

Saraste, M., Sibbald, P., and Wittinghofer, A. (1990) The P-loop: a common motif in ATP- and GTP-binding proteins. *Trends Biochem Sci* **15:** 430–434.

Senior, B.W. (1983) *Proteus morgani* is less frequently associated with urinary tract infections than *Proteus mirabilis* – an explanation. *J Med Microbiol* **16:** 317–322.

Senior, B., Loomes, L., and Kerr, M. (1991) The production and activity *in vivo* of *Proteus mirabilis* IgA protease in infections of the urinary tract. *J Med Microbiol* **35:** 203–207.

Stern, A., Brown, M., Nickel, P., and Meyer, T. (1986) Opacity genes in *Neisseria gonorrhoeae*: control of phase and antigenic variation. *Cell* **47:** 61–71.

Svanborg-Eden, C., Larson, P., and Lomberg, H. (1980) Attachment of *Proteus mirabilis* to the human urinary sediment epithelial cell *in vitro* is different from that of *Escherichia coli*. *Infect Immun* **27:** 804–807.

Swihart, K., and Welch, R. (1990a) The HpmA hemolysin is more common than HlyA among *Proteus* isolates. *Infect Immun* **58:** 1853–1860.

Swihart, K., and Welch, R. (1990b) Cytotoxic activity of the *Proteus* hemolysin HpmA. *Infect Immun* **58:** 1861–1869.

Walker, J., Saraste, M., Runswick, M., and Gay, N. (1982) Distantly related sequences in the alpha and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* **1:** 945–951.

Wandersman, C., and Delepelaire, P. (1990) TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. *Proc Natl Acad Sci USA* **87:** 4776–4780.

Wassif, C., Cheek, D., and Belas, R. (1995) Molecular analysis of a metalloprotease from *Proteus mirabilis*. *J Bacteriol* **177:** 5790–5798.