The Secreted Hemolysins of *Proteus mirabilis*, *Proteus vulgaris*, and *Morganella morganii* Are Genetically Related to Each Other and to the Alpha-Hemolysin of *Escherichia coli*

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Secreted hemolysins were extremely common among clinical isolates of *Proteus mirabilis*, *Proteus vulgaris*, and *Morganella morganii*, and hemolytic activity was either cell associated or cell free. Southern hybridization of total DNA from hemolytic isolates to cloned regions of the *Escherichia coli* alpha-hemolysin (hly) determinant showed clear but incomplete homology between genes encoding production of hemolysins in the four species. One of the two *E. coli* secretion genes, hlyD, hybridized only with DNA from *P. vulgaris* and *M. morganii*, which produced cell-free hemolysis, but not with that from *P. mirabilis*, which showed only cell-associated activity. Molecular cloning of the genetic determinants of cell-free hemolytic activity from *P. vulgaris* and *M. morganii* chromosomal DNA allowed their functional analysis via inactivation with the transposons Tn1000 and Tn5. Both hemolysin determinants were about 7.5 kilobase pairs and comprised contiguous regions directing regulation, synthesis, and specific secretion out of the cell. Transposon mutations which eliminated secretion of the *Proteus* and *Morganella* hemolysins could be complemented specifically by the *E. coli* hemolysin secretion genes hlyB or hlyD. Alignment of the physically and functionally defined hly determinants from *P. vulgaris* and *M. morganii* with that of the *E. coli* alpha-hemolysin confirmed a close genetic relationship but also indicated extensive evolutionary divergence.

Production of cytoxic hemolysins is common among both gram-negative and -positive pathogenic bacteria, but its molecular basis varies greatly (13). In *Escherichia coli*, secretion of the active hemolysin protein demands translocation across both cytoplasmic and outer membranes, and it is now evident that the process involved does not, in contrast for example to the transport of enterotoxins (25, 27), make use of a conventional N-terminal signal sequence on the secreted protein (5) nor does it proceed via a cell lysis analogous to that which releases effect of colicin E (16).

This novel secretion is absolutely dependent on two proteins, HlyB and HlyD, which are encoded by the four-gene hemolysin (hly) determinant itself (2, 12, 21, 22, 29), and it seems possible that specific export is achieved by the two secretory proteins spanning both membranes to promote recognition and direct translocations of the 107-kilodalton hemolysin molecule (9, 22). It nevertheless remains unclear how this might occur, and although the C terminus of the hemolysin protein (HlyA) is now known to be essential (9), little is known of the sequences intimately involved in recognition and interaction during secretion or indeed in coordinating synthesis and secretion. The secretion protein HlyB has at least one potential ATP-binding domain analogous to those present in a wide range of kinases (3, 7) and bacterial transport proteins (15), provoking the view that this feature is central to the active secretion of hemolysin.

Both the organization (4, 18, 20, 21, 24, 28, 30) and nucleotide sequence (6, 14) of the four contiguous hemolysin genes (hlyC, hlyA, hlyB, and hlyD) are rigidly conserved on both plasmids and chromosomes throughout *E. coli* strains. The genes nevertheless have a G+C content of about 39% rather than 50%, the percentage typical of the *E. coli* genome, and this suggests that the hly genes did not originate in this species. We report here on their relationship to the genetic determinants of hemolysin production in *Proteus mirabilis* and *Proteus vulgaris*, both of which have 39% G+C genomic DNA, and also in *Morganella* (formerly *Proteus*) *morganii*, which has a genomic G+C content of 50%, analogous to that of *E. coli*.

**MATERIALS AND METHODS**

**Bacteria.** Clinical isolates of *P. mirabilis*, *P. vulgaris*, *M. morganii*, and *E. coli* were obtained from the United Kingdom (Ninewells Hospital, Dundee, and Charing Cross Hospital, London), Hungary (University Hospital, Budapest), and the Federal Republic of Germany (University Hospital, Würzburg). Representative isolates cited in the text are listed in Table 1. Typing of the strains was performed as described previously (26).

**Hemolysin characterization.** Hemolytic activity was assayed either on brain heart infusion agar containing 2% washed horse erythrocytes (and 100 μg of p-nitrophenylglycerine per ml [Sigma] when swarming was evident) or, after growth in brain heart infusion broth, in a 2% erythrocyte suspension in 0.85% NaCl plus 20 mM CaCl₂ incubated at 40°C for 15 min. Activity was measured by the release of hemoglobin (A₄₁₅) and defined as cell free, cell associated, or intracellular after comparative assay of intact bacterial culture, supernatant fluid from a centrifuged culture, and cleared cell extract of washed (10 mM Tris chloride, pH 7.4) bacterial cells (which were sonicated twice [20 s each time] on ice in an ultrasonic disintegrator [MSE Scientific Instruments] and centrifuged for 5 min at 1,500 × g).

**Cosmid cloning, subcloning, restriction mapping, and transformation.** Total cellular DNA was isolated as described previously (23), and partially digested *Sau*3A frag-
membrane of 20 to 35 kilobase pairs (kb) were isolated from NaCl gradients (13 ml; 1.25 to 5 M) run for 3.5 h at 32.000 rpm and 18°C in an SW 40 rotor. Ligation into BamHI-cut cosmid pHC79 was followed by in vitro packaging (Boehringer) and transfection of E. coli HB101. Recombinant cosmid DNA from hemolytic colonies was subjected to preliminary restriction nuclease analyses and was then subcloned as HindIII or PsI partial fragments into plasmid vector pBR325. Restriction endonuclease analyses, deletion mapping, gel electrophoresis, and transformation of E. coli HB101 were performed as described (23).

Transposon mutagenesis. Insertional inactivation of hly recombinant plasmids was achieved with Tn1000, isolated after conjugation of cointegrates formed with F::Tn1000 (11), and also Tn3, delivered by bacteriophage lambda (1, 17). Mapping of insertions followed comparative restriction nuclease analyses.

Southern DNA-DNA hybridization analyses. Total cellular DNA (15 µg) was digested to completion overnight with 10 U of restriction endonuclease per µg, and fragments were separated in 0.8% agarose before transfer to nitrocellulose filters (Schleicher & Schuell). Prehybridization and hybridization were performed at 42°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate–50% formamide–100 µg of denatured salmon sperm DNA per ml. Prehybridization was carried out for 1 h, and hybridization was carried out overnight. After hybridization, filters were washed three times for 1 h in low-stringency conditions (i.e., at 50°C in 0.1× SSC–0.1% SDS) and dried. Autoradiography took up to 7 days at −70°C with intensifying screens. DNA probes were isolated from low-melting agarose (Bio-Rad) and labeled by nick translation with [α-32P]dCTP (3,000 Ci/µmol; Amersham).

Complementation tests. Recombinant DNA derived from pBR325 and containing either Tn5 or Tn1000 was introduced by transformation into E. coli HB101 carrying incompatible recombinant plasmids derived from the vector pACYC184 and carrying, singly or together, hlyB and hlyD from E. coli (21). Selection of the incoming plasmids was effected by the presence of antibiotics. That both recombinant plasmids were maintained independently in the recA host was confirmed by plasmid DNA isolation from the hemolytic double transformants, retransformation of E. coli HB101, and selection for the transposon-bearing plasmid. No hemolytic transformants were observed.

RESULTS

Hemolysin production among Proteae clinical isolates. Ninety-five strains of the Proteae tribe were examined, and these were isolated from a wide range of clinical specimens in four hospitals. There was a high incidence of hemolysin production among the three species; 94% of P. mirabilis, 84% of P. vulgaris, and 56% of M. morganii strains were clearly hemolytic when grown in brain heart infusion broth. The isolates taken for further examination appeared to be typical and are listed in Table 1.

Hemolysin production during bacterial batch growth. Four independent isolates each of P. mirabilis, P. vulgaris, and M. morganii and two of E. coli (including the representative isolates listed in Table 1) were grown in brain heart infusion broth, and at intervals throughout batch growth they were assayed for extracellular (both cell-associated and cell-free) and intracellular hemolytic activities. This was achieved by incubating washed erythrocytes with samples of the whole culture, supernatant from a centrifuged culture, or sonicated washed cells.

All strains tested synthesized and secreted active hemolysin in the logarithmic growth phase, and all showed a dramatic loss of hemolytic activity in the late logarithmic phase. No intracellular activity was detected as the cells entered stationary phase, but extracellular activity sometimes persisted, slowly declining. Typical activities are shown in Fig. 1. Although some variation was seen in the maximum activities attained by isolates of a particular species, the large differences depicted among the four species were a clear and regular feature, with the members of the Proteae being more hemolytic than were the two E. coli strains examined (this was seen not only with maximal intracellular levels but also with extracellular activity).

Two types of hemolytic activity. There were two types of secreted hemolytic activity. That of all the M. morganii strains was seen to equal extents in both whole culture and cell-free assays. This hemolysis was visible around bacterial colonies and was reminiscent of E. coli hemolysis. That of all P. mirabilis strains was seen (at high levels) only in whole-culture assays: i.e., activity was secreted but still associated with the cells (this was not seen on blood agar plates around growing colonies). Both these types of activity were produced by isolates of P. vulgaris, the majority (ca. 60%) showing cell-associated activity. Activity of the α-hemolysin of uropathogenic E. coli is dependent on the presence of Ca2+ in the erythrocyte assay, whereas others (e.g., the gram-positive cytolysins) are not (12, 13). The two types of hemolytic activity produced by members of the Proteae differed in this respect; the cell-free activity of M. morganii and normally that of P. vulgaris were strictly dependent on the presence of Ca2+ (maximal activity was attained with 10 mM Ca2+), and no hemolysis was seen when EDTA was added to the assay. In contrast, the cell-bound activity typical of P. mirabilis and P. vulgaris was not influenced by the addition of 20 mM Ca2+ or 10 mM EDTA; i.e., the activity is Ca2+ independent (Table 1).

Southern DNA-DNA hybridization analyses. Hybridization
of nick-translated *E. coli* alpha-hemolysin gene probes with *HindIII*-cleaved total DNA from the representatives of each species cited above was performed at low salt concentration. The restriction nuclease fragments used as probes cover most of the *hly* determinant (12, 14) and are depicted in Fig. 2. The hybridization results (Fig. 2) indicate significant homology, but this was only seen with relatively high amounts of total DNA (15 μg per track), high levels of specific activity, and 3 to 7 days of autoradiography. The intensity of hybridization was about 1/20 of that seen when total DNA was taken from *E. coli* strains, e.g., strain 519.

All three *Proteus* DNA hybridized reproducibly in a number of *HindIII* fragments with the 3,650-base-pair (bp) *BamHI*-BglII fragment (probe 1). This covers 2,490 bp of the 3,074-bp *hlyA* hemolysin gene and also 150 bp of the 507-bp *hlyC* gene, which, together with *hlyA*, determines active intracellular hemolytic activity in *E. coli*. This may indicate that these strains carry more than one copy of this region. Strains of *P. mirabilis* and *P. vulgaris* showed a similar pattern of hybridization which was quite different from that of *M. morganii*. No extra hybridization was obvious with the 3,310-bp *EcoRI*-EcoRI fragment (probe 2) carrying 970 bp of (3') *hlyA*, the entire 2,123-bp secretion gene *hlyB*, and 115 bp of *hlyD*, the second secretion gene. Again, several fragments hybridized. In contrast, hybridization with the 840-bp *HindIII*-PstI probe fragment (probe 3) carrying only the central region of the 1,436-bp *hlyD* gene was found in only one *HindIII* fragment of DNA from *P. vulgaris* and *M. morganii*, and no homology was detected with DNA from *P. mirabilis*.

**Isolation of *hly* determinants from *P. vulgaris* and *M. morganii*.** *P. vulgaris* 76362 and *M. morganii* 227 producing cell-free hemolysins appeared to carry no extrachromosomal DNA (data not shown). Cosmid cloning was therefore used to isolate the genetic determinants of hemolysins secreted by these strains. Several hemolytic colonies were obtained after cloning of partial *Sau3A* fragments into the cosmid vector pHC79 and transfection of packaged recombinant cosmid DNA into *E. coli* HB101. Transfections derived from the same strain showed no significant differences in hemolytic phenotype on blood agar and had very similar restriction patterns. Recombinant DNA was isolated from representatives of the two types (pcos763-7 and pcos227-5, respectively) and subcloned via *HindIII* and *PstI* partial digests into the plasmid vector pBR325.

Recombinant pBR325 plasmids from hemolytic *E. coli*...
HB101 transformants were isolated and digested with either HindIII or PstI, and one of the smallest representatives of each was taken for detailed analysis. These were pVU763-710 and pMO227-508, which were stable and carried inserts of 8.6 and 9.6 kb, respectively.

**Physical and functional comparison of the hly determinants.**

*E. coli* HB101 strains carrying each of the recombinant DNAs pVU763-710 and pMO227-508 were subjected to mutagenesis with the transposons Tn5 and Tn1000. From each strain, 40 to 50 insertion mutants were obtained which were nonhemolytic on blood agar, and these were checked for residual hemolytic activity, particularly after cell sonication. In each case, these mutants were of two kinds: those lacking all activity, secreted or intracellular, and those which retained normal (or slightly elevated) intracellular activity while having lost all extracellular activity. These findings suggested a specific separation of synthesis and secretion encoded by both determinants.

Detailed restriction nuclease maps of the cloned hly determinants pVU763-710 and pMO227-508 were constructed and were used to map the sites of transposon insertions. These defined the extent of the regions coding for synthesis and secretion and allowed (Fig. 3) an alignment of the two hemolysin determinants with each other and also the previously published *E. coli* alpha-hemolysin determinant of the recombinant DNA pANN202-312 (12, 14).

Full intracellular activity was determined in both *Proteae* genera by a region of ca. 4.2 kb, and this was followed immediately by ca. 3.3 kb dictating secretion of the active hemolysin. In addition, several Tn5 and Tn1000 insertions (occurring at a frequency of about 1% of the Hly- mutant phenotype) caused considerable increases in the hemolytic activity of both determinants. They mapped very closely to each other at the end of the cloned determinants and adjacent to the region encoding synthesis of active intracellular hemolysin. These mutated (hyp) loci aligned approximately with the regulatory sequences of the *E. coli* hlyC-hlyA-hlyB transcriptional unit and will be described in detail elsewhere.

Alignment of the regions dictating synthesis and secretion allowed a comparison of restriction nuclease sites throughout the three hly coding regions. Of the 26 restriction nuclease sites mapped within the *E. coli* determinants, 7 were apparent in one of the *Proteae* hly determinants, and 4 were apparently shared by both. Comparison of the two *Proteae* determinants revealed 5 of the 25 restriction nuclease sites in the hly determinant of pVU763-710 to be present in pMO227-508. This restriction nuclease site correspondence clearly suggests basic identity between the three hly determinants. Nevertheless, the degree of nuclease site coincidence is not comparable to that apparent between independently isolated *E. coli* hly determinants; e.g., the...
pANN202-312 hly determinant (originally plasmid borne [12]) and the cloned E. coli chromosomal hly determinant of recombinant DNA pSF4000 (6, 30) share 24 of the 26 sites cited (Fig. 3).

The contiguous regions encoding the synthesis and secretion of the two hemolysins are comparable in size to each other and to the hly determinant of E. coli, and their close organizational similarity is further indicated by two more points. Polar Tn5 insertions in the ca. 3.3-kb secretory region had no effect on the level of intracellular activity, indicating that secretory functions are transcribed independently or distal to those determining synthesis. A similar transcriptional organization is also indicated by the alignment of the hyp mutations in both Proteaeae determinants with the transcription initiation region of the pANN202-312 hlyC-hlyA-hlyB transcriptional unit.

Revision of the Southern hybridization data of Fig. 2 in the light of the maps supports these contentions. Homology in one P. vulgaris 1.4-kb HindIII fragment when probed with E. coli probe 2 correlated with the presence of such a fragment aligned with the central part of E. coli hlyA. This was also seen when the overlapping probe 1 was hybridized, and the stronger hybridization in this instance could be expected because of the additional 1.4-kb HindIII fragment spanning the 5’ end of hlyA and the 3’ half of hlyC. Similarly, the hybridization of a ca. 3.8-kb HindIII fragment with both probes (2 and 3) spanning the downstream secretion genes of E. coli was consistent with the alignment of such a fragment in the P. vulgaris determinant. However, the observed hybridization of a HindIII fragment of comparable size (ca. 3.8 kb) to probe 1 was not predicted from the map of P. vulgaris hly, and such additional hybridization was also evident with probe 2 (i.e., hybridization was seen with a double band of this size). This indicates homology with E. coli hly in a further region not isolated on the recombinant DNA pVU763-710. Reexamination of the hybridizations to the HindIII-cut total cellular DNA of M. morganii revealed similar features. A fragment in the region of the 6.5-kb-size marker hybridized to all three E. coli probes and corresponded to the right-hand HindIII fragment of pVU763-710. Nevertheless, after allowing for the hybridization with the unknown HindIII fragment (>2.5 kb) which includes the region to the left of the single HindIII site in M. morganii hly, there is still the 1.4-kb HindIII fragment which shows homology and which is clearly not present in pMO227-508.

Table 2. Complementation of Proteaeae secretion mutations

<table>
<thead>
<tr>
<th>Mutant plasmid* (Proteaeae genes)</th>
<th>pLG579 pLG595 pLG575 pACYC184</th>
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<tbody>
<tr>
<td>pVU763-710/T35 (hlyB: Tn1000)</td>
<td>+ - + -</td>
</tr>
<tr>
<td>pVU763-710/T4 (hlyD: Tn1000)</td>
<td>- + - -</td>
</tr>
<tr>
<td>pMO227-205/T11 (hlyB: Tn3)</td>
<td>+ - + -</td>
</tr>
<tr>
<td>pMO227-205/T3 (hlyD: Tn3)</td>
<td>- + + -</td>
</tr>
</tbody>
</table>

*Plasmids, derived from pBR325, bearing secretion-defective hly determinants from P. vulgaris or M. morganii. The Proteaeae genes inactivated were putatively designated hlyB or hlyD after alignment of the three hly determinants (Fig. 3).

**Complementation by plasmid**: +, complementation restored the secreted activity to the level determined by the parent nonmutated recombinant plasmids; −, no change in the secretion-negative phenotype determined by the Proteaeae recombinant plasmids; i.e., they retained intracellular activity only.

The cytotoxic alpha-hemolysin (HlyA) of E. coli is secreted across both gram-negative membranes without the aid of an N-terminal hydrophobic signal sequence (5). This is effected by a specific envelope transport system formed by two proteins, HlyB and HlyD, which are encoded by genes of the contiguous four-gene hemolysin determinant (12, 21, 28, 30). Examination of the highly conserved hly determinant sequence in E. coli has revealed surprisingly that the four genes have a G+C content of 39% rather than the expected 50% of the genome (6, 14). This suggested to us a possible common ancestry with the uncharacterized hemolysin determinants of Proteus, a genus of the family Enterobacteriaceae having this low G+C genomic content. To investigate this possibility and open the way to further elucidation of the secretion mechanism, we examined the phenotypes and genotypes of hemolysins from three different species of Proteaeae.

The synthesis of active intracellular hemolysin during batch growth follows a similar pattern in P. vulgaris, P. mirabilis, M. morganii, and E. coli, although hemolysin activity in the 12 isolates of Proteaeae was considerably greater than that seen in the two strains of E. coli tested. Maximal activity was attained in the mid-logarithmic phase and rapidly decayed as culture growth ended. This sharp pulse was more pronounced in the Proteaeae strains. Se-
creted activity in all species followed closely that seen intracellularly, although activity often persisted longer, presumably resulting from the formation of more stable complexes (12). Extracellular hemolytic activity was cell free in the case of all M. morganii and roughly 40% of the P. vulgaris strains, but in all P. mirabilis isolates and the remaining P. vulgaris strains, activity was found only in association with the intact bacterial cells. The possibility that the different types of hemolysin detected in the various isolates were fundamentally the same but differed in their secretary capacity was supported by hybridization of total cellular DNA to regions of the E. coli alpha-hemolysin determinant. Homology was evident with DNA from strains that produced both types of activity, but hybridization was in every case considerably lower than that seen with total DNA of wild-type hemolytic E. coli. Although DNA from P. mirabilis, P. vulgaris, and M. morganii all hybridized with E. coli probes spanning hlyA, the hemolysin structural gene, and hlyB, the principal secretion gene, when probing was performed with hlyD, the second transport gene, no homology was seen with P. mirabilis DNA. Hybridization occurred with a number of restriction endonuclease fragments which were not observed in the hly recombinant DNA, suggesting that Proteaeae isolates may perhaps carry more than one hly determinant. In E. coli, such multiple copies are associated with specific insertion elements, which are also implicated in the evolution of virulence gene clusters (19).

Molecular cloning of total cellular DNA from P. vulgaris 76362 and M. morganii 227 (both of which produce cell-free hemolytic activity) gave a greater insight into the relationship of the hly genes to each other and to those of E. coli. In both cases, the region needed for hemolysin synthesis and secretion was ca. 7.5 kb, and insertional inactivation with transposons Tn5 and Tn1000 revealed that, analogous to E. coli hly, each determinant consisted of two contiguous stretches of ca. 4.2 and 3.3 kb encoding, respectively, the synthesis of active hemolysin and its secretion out of the cell. That these analogous functions are dictated by closely related genes is indicated by the coincidence of several restriction nuclease sites in the three hly determinants when aligned on the basis of transposon mutant phenotypes.

In addition, transposon mutations which caused a loss of secretion function in the two Proteaeae hly determinants could be complemented by the presence of functional E. coli secretion genes hlyB and hlyD. Moreover, complementation occurred only when the E. coli secretion gene present, hlyB or hlyD, was that corresponding to the region mutated in the aligned Proteus or Morganella determinant. This points to a close functional relatedness between the components of the secretion systems, not only the secretion proteins but also the relevant signal(s) on the hemolysin proteins.

Nevertheless, about two-thirds of the restriction sites were unique to a single determinant, and only about 15% were common to all three hly determinants. This presents a great contrast to the high degree (about 95%) of restriction site homology seen between hly determinants from different isolates of E. coli. The data also indicate that the hlyD gene of E. coli is absent from P. mirabilis but present in the other two species, and this could explain the production by P. mirabilis strains of secreted hemolytic activity which remains associated with the cell and is not released into the medium. This seems to be an attractive possibility because in E. coli the hlyD gene is believed to be transcribed separately from the hlyC-hlyA-hlyB transcriptional unit (18, 21) and therefore may not be a constant presence in enterobacterial hly determinants. This might indicate that the protein HlyD can be substituted by a different protein to form a critical secretory channel with HlyB, or alternatively, HlyB may be able to function independently to a large extent in the secretion process.

The data suggest that the Proteaeae and E. coli hly determinants are closely related but have diverged significantly. In view of the G+C content of the E. coli hly genes (39%), it seems possible that they may have originated in Proteus spp. and spread to E. coli and M. morganii. We hope that the comparison of conserved and divergent protein sequences will help define regions and interactions of critical importance in the novel secretion of enterobacterial hemolysins. Recent reports (8, 10) of striking identity between E. coli HlyB and the mammalian p-glycoprotein which confers multiple drug resistance on tumor cells suggest that such studies may also have relevance to eucaryotic export.

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LITERATURE CITED


