Iron-repressible Outer-membrane Proteins of Pasteurella haemolytica

By HARRY G. DENEER1* AND ANDREW A. POTTER2

1BIOSTAR, Inc., Box 1000, Sub PO 6, Saskatoon, Saskatchewan, Canada S7N 0W0
2Veterinary Infectious Disease Organization, University of Saskatchewan, 124 Veterinary Road, Saskatoon, Saskatchewan, Canada S7N 0W0

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The outer-membrane protein (OMP) profile of Pasteurella haemolytica grown under iron-replete and iron-restricted conditions was studied by polyacrylamide gel electrophoresis and immunoblotting. A serotype 1 isolate induced the synthesis of a new 77000 M, OMP in iron-restricted media while two other proteins of 100000 M, and 71000 M, were synthesized in increased amounts. None of these proteins were peptidoglycan-associated or heat-modifiable, and only the 100000 M, protein showed some degree of disulphide cross-linking. Kinetic analysis revealed that the iron-repressible proteins appeared in the outer membrane within 15 min of establishment of iron-restricted conditions. Analysis of P. haemolytica isolates representing serotypes 1 to 12 showed that iron-repressible OMPs of 77000 M, and 71000 M, could be induced in all 12 serotypes but that there was some variability in the expression of the 100000 M, protein. Immunoblotting of OMPs with convalescent sera from P. haemolytica-infected calves indicated that antibodies directed against all three iron-repressible OMPs were present, suggesting that these proteins were expressed in vivo.

INTRODUCTION

Bovine pneumonia pasteurellosis, or shipping fever pneumonia, is a severe, contagious, often fatal respiratory disease in cattle (Yates, 1982). The aetiology of this disease is poorly understood as it involves a complex interaction between viral, bacterial and environmental factors, but it is clear that the primary bacterial pathogen is Pasteurella haemolytica biotype A, serotype 1 (Babiuk & Acres, 1984). As a consequence, much effort has gone into identifying the virulence factors elaborated by this organism. While components such as pili (Morck et al., 1987), capsule (Adlam et al., 1984), and a leukotoxin (Shewan & Wilkie, 1982) have been well studied, little attention has been focused on bacterial outer-membrane protein (OMP) antigens, especially those elaborated only in vivo.

Studies with other bacterial pathogens have established that the ability to acquire iron in vivo is an important factor in their pathobiology (Bullen, 1981; Finkelstein et al., 1983). In addition to being an essential bacterial nutrient, iron can modulate the synthesis of such virulence factors as haemolysin (Holmes & Russel, 1983) and exotoxin (Lory, 1986), and may be critical to the efficient production of leukotoxin by P. haemolytica (Gentry et al., 1986). While iron is plentiful in vivo, it is largely unavailable for bacterial use because it is complexed with iron-binding glycoproteins such as transferrin in serum and lactoferrin on mucosal surfaces (Aisen & Listowski, 1980; Bezkorovainy, 1981). To overcome this natural iron-restriction, most pathogens express high-affinity iron-uptake systems that can compete with transferrin and lactoferrin for iron. Such uptake systems usually involve two components; low-M, siderophores which chelate iron, and iron-repressible outer-membrane proteins (IROMPs) which function as receptors for the iron–siderophore complexes (Braun, 1985; McIntosh et al., 1979; Neilands, 1989).

Abbreviations: OMP, outer-membrane protein; IROMP, iron-regulated outer-membrane protein.
passively immunized with antibodies against the IROMPs of Finkelstein, 1983), while antibodies directed against IROMPs are present in the convalescent sera of infected patients (Fohn et al., 1987). Bolin & Jensen (1987) have shown that turkeys passively immunized with antibodies against the IROMPs of Escherichia coli are partially protected against E. coli septicaemia. Therefore, IROMPs may have potential as protective antigens by inducing antibodies which block siderophore-mediated iron uptake and/or which promote phagocytosis.

The purpose of the present study was to determine whether P. haemolytica expressed IROMPs when grown under iron-limited conditions, whether such proteins were common to all serotypes, and if antibodies directed against IROMPs were present in calves infected with P. haemolytica.

**METHODS**

**Bacterial strains.** Type strains of P. haemolytica biotype A, serotypes 1–12, were obtained from the American Type Culture Collection. Strain B122 was a serotype 1 clinical isolate originally cultured from the lung of a calf which died of pneumatic pasteurellosis. To avoid repeated subculturing, samples of infected lung tissue were frozen at −70 °C and P. haemolytica was isolated by streaking on Blood Agar plates when required.

**Growth conditions.** Iron-restricted growth was achieved by growing bacteria in Trypticase Soy broth (TSB; Gibco Laboratories) supplemented with the iron chelator 2,2'-dipyridyl (Sigma) to a final concentration of 200 μM (TSB-D), or in some experiments, with 200 μM iron-free (Rogers, 1973) ethylenediaminedihydroxyphenylacetic acid (EDDA; Sigma). Initially, an exponential-phase culture in TSB was diluted 1:500 in TSB plus chelator and grown for 12–18 h with aeration at 37 °C. However, this method resulted in only low yields of cells. As an alternative, P. haemolytica strains were grown at 37 °C in TSB for 2–3 h until an OD₆₆₀ of 0.2–0.3 was reached. At this time, 2,2'-dipyridyl or EDDA was added to the final concentrations indicated above and growth continued for an additional 3 h. The OMP profiles of strains grown by the above two methods were identical and consequently the latter method was used for most experiments. Iron-replete bacteria were grown in TSB alone or in TSB-D supplemented with 50 μM-Fe(NO₃)₃.

For time-course experiments, P. haemolytica strain B122 was grown in TSB to an OD₆₆₀ of 0.1. A sample of these cells was removed and 2,2'-dipyridyl (200 μM) was added to the remainder of the culture. Growth was continued at 37 °C and OD₆₆₀ measurements were made at appropriate intervals. Samples of cells were removed at 15 min intervals, rapidly chilled, and immediately collected by centrifugation (6000 g, 10 min). Cell pellets were frozen at −20 °C and used within 24 h for the preparation of outer membranes.

**Preparation of OMP-enriched fractions.** Outer membranes were prepared by the method of Barenkamp et al. (1981) with some modifications. Cells were suspended in 10 mM-HEPES buffer (pH 7·4) and sonicated for 45 s (Braunsonic 1510, intermediate probe, 60 W). Debris and unbroken cells were pelleted by centrifugation at 3000 g for 10 min and the supernatant was added to one-quarter volume of 2% (w/v) Sarkosyl (sodium N-lauroyl sarcosinate; Sigma) and incubated at room temperature for 10 min. The partially purified outer membrane fraction was pelleted by centrifugation at 10000 g for 1 h, resuspended in 10 mM-HEPES buffer and again treated with 1 vol. of 2% Sarkosyl for 20 min at room temperature. This procedure yielded an outer membrane fraction of greater purity than could be obtained with a single Sarkosyl extraction. The final Sarkosyl-insoluble, OMP-enriched fraction was sedimented by centrifugation as above and the pellet was suspended in 10 mM-HEPES buffer before storage at −20 °C.

**Polyacrylamide gel electrophoresis and immunoblotting.** Proteins were separated by discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% (w/v) stacking gel and separating gels of 8 or 12% (Laemmli, 1970). The gels were solubilized by treatment at 100 °C for 7 min in the presence of 1 vol. of sample buffer [2·5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 25% (v/v) glycerol, 0·003% (w/v) bromophenol blue, in 0·05 M-Tris/HCl pH 6·8]. Protein bands were visualized by staining with Coomassie Brilliant Blue R250 (Bio-Rad).

Immunoblotting was performed essentially as described by Towbin et al. (1979). Gels were soaked in transfer buffer [2 mm-Tris base, 192 mm-glycine, 0·03% (w/v) SDS, 20% (v/v) methanol] for 30 min prior to electroblotting onto nitrocellulose membranes (0·45 μm pore size; Bio-Rad). Electrophoretic transfer was achieved with a Bio-Rad Transblot or Mini-Transblot cell using the conditions recommended by the manufacturer. Non-specific binding sites were blocked by incubating the nitrocellulose for 1 h in TST buffer [10 mm-Tris/HCl, 150 mm-NaCl, 0·05% (v/v) Tween-20, pH 7·5] containing 4% (w/v) bovine serum albumin. Membranes were then incubated for 1 h with antisera diluted 1:500 in TST buffer. After three 10 min washes with TST buffer, membranes were reacted for 1 h with alkaline-phosphatase-conjugated goat anti-bovine immunoglobulin G (heavy- and light-chain specific; Kirkegaard & Perry Laboratories) in TST buffer. Membranes were again washed with TST buffer and developed with 5-bromo-4-chloro-3-indolyl phosphate: nitroblue tetrazolium (Bio-Rad) substrate. Reactions were stopped by washing the nitrocellulose membranes with water.
Antisera were derived from calves which had been experimentally infected with *P. haemolytica* B122 (A. A. Potter, unpublished results) or from calves which had survived a natural *P. haemolytica* serotype 1 infection.

**RESULTS**

**Effects of iron-restriction on the OMP composition of *P. haemolytica***

To assess the effects of iron-restriction on OMPs, *P. haemolytica* strain B122 was grown in TSB containing the iron chelator 2,2'‑dipyridyl. OMPs were compared by SDS-PAGE with the OMPs from cells grown in TSB with dipyridyl but supplemented with Fe(NO₃)₃ to overcome the iron-restriction (Fig. 1). A number of differences were apparent among the high-Mₘ OMPs. Two proteins of apparent Mₘ 100000 and 71000 were barely detectable in cells grown under iron-replete conditions but were present in increased amounts in iron-restricted cells. In addition, a new protein of 77000 Mₘ could be seen in iron-restricted but not in iron-replete cells. Interestingly, growth in iron-restricted media appeared to cause a reduction in the amount of a 97000 Mₘ OMP when compared with iron-replete cells. No other OMP changes were consistently evident after iron-restriction, even on gels which had been silver stained (data not shown).

To ensure that the changes in the 100000, 77000 and 71 000 Mₘ OMPs were not the result of generally poor growth conditions or of the particular iron chelator used, *P. haemolytica* B122 was grown under several different conditions and the OMP profile examined as before. For these experiments, the medium was TSB containing sufficient iron for growth. The generation time of the cells was increased by reducing the aeration of the culture (50 r.p.m. versus 180 r.p.m.). Fully aerated cells were also harvested at the mid-exponential, late-exponential or stationary phase of growth prior to outer membrane preparation. Finally, an aerated culture was grown to mid-exponential phase, then aeration was stopped for 3 h prior to harvesting the cells. While there were minor changes evident among the lower-Mₘ OMPs under some of these conditions (see for example, Fig. 1, lane 2), in no case was the induction of the 100000, 77000 or 71 000 Mₘ iron-repressible proteins observed. Finally, we induced iron-restricted conditions by supplementing TSB with a different iron chelator, EDDA, and grew *P. haemolytica* B122 as before. In this case, the induction of the three IROMPs was identical to that seen for cells grown in the presence of 2,2'-dipyridyl (data not shown).

**Kinetics of IROMP induction**

Cultures of *P. haemolytica* B122 in TSB were grown in parallel and to one was added 2,2′-dipyridyl to induce iron-restriction. Samples of this culture were removed at intervals and examined for OMP composition. Growth of cells started to deviate from exponential within 30 min of addition of the iron chelator and appeared to stop completely after about 120 min (Fig. 2a). Analysis of outer membranes (Fig. 2b) indicated that induction of the 100000, 77000 and 71 000 Mₘ IROMPs occurred within 15 min of 2,2′-dipyridyl addition and reached a maximum after 30–45 min of growth in iron-restricted media.

**Physical characterization of IROMPs**

In order to examine the physical properties of the *P. haemolytica* IROMPs, outer membranes were subjected to various solubilization conditions prior to SDS-PAGE (Fig. 3). The three IROMPs did not appear to be heat-modifiable or peptidoglycan-associated since solubilization in SDS sample buffer at different temperatures and for different lengths of time did not alter the apparent electrophoretic migration of these proteins. Omission of 2-mercaptoethanol from the SDS solubilization buffer had no effect on the migration of the 77000 Mₘ and 71 000 Mₘ IROMPs, but the 100000 Mₘ protein showed a slightly increased mobility (Fig. 3, lane 6), suggesting some degree of disulphide cross-linking. Although a new protein of about 120000 Mₘ was seen under non-reducing conditions, it is unlikely that this is related to the 100000 Mₘ IROMP since studies with iron-replete cells have shown that a protein of 120000 Mₘ appears concomitantly with the disappearance of a 41 000 Mₘ major OMP under these solubilization conditions (H. G. Deneer, unpublished results).
Fig. 1. Response of \textit{P. haemlytica} B122 to iron restriction. OMPs were separated on an SDS-polyacrylamide (12\%) gel and stained with Coomassie blue. Lane 1, \textit{M}, standards. Growth conditions for subsequent preparations were as follows: lane 2, aerobically grown cells in TSB allowed to stand without aeration for 3 h prior to harvesting; lane 3, aerobically grown cells in TSB plus 2,2'-dipyridyl with 50 \,\mu M \text{Fe(NO}_3\text{)}_3 added (iron-replete); lane 4, aerobically grown cells in TSB plus 2,2'-dipyridyl (iron-restricted). Arrows indicate the positions of the 100000, 77000 and 71000 \textit{M}, iron-repressible proteins in lane 4.

Fig. 2. Kinetics of IROMP induction. (a) Growth of \textit{P. haemolytica} B122 in TSB was monitored by measuring OD\textsubscript{600}. ■, Culture to which 2,2'-dipyridyl was added (to induce iron-restriction) at the time indicated by the arrow; ○, culture without additions (iron-replete). (b) Samples for OMP preparation were removed at 15 min intervals and proteins were separated on an SDS-polyacrylamide (8\%) gel. Lane 1, OMPs from cells harvested 1 min prior to 2,2'-dipyridyl addition; lane 2, 15 min post-addition; lane 3, 30 min post-addition; lane 4, 45 min post-addition; lane 5, 60 min post-addition; lane 6, 75 min post-addition. Arrows indicate the positions of the 100000, 77000 and 71000 \textit{M}, IROMPs.

\textbf{Serotype-specific differences in \textit{P. haemolytica} IROMPs}

At least 15 serotypes of \textit{P. haemolytica} have been identified on the basis of differences in soluble or extractable surface antigens (Biberstein \textit{et al.}, 1960; Fraser \textit{et al.}, 1982). We therefore wished to determine whether differences might also exist in the number or size of IROMPs in \textit{P. haemolytica} of different serotypes. Reference strains of \textit{P. haemolytica} representing serotypes 1 to 12 were grown in TSB with or without 2,2'-dipyridyl and their OMPs were examined by SDS-
Fig. 3. Effect of solubilization conditions on SDS-PAGE mobility of *P. haemolytica* IROMPs. Lane 1, 37 °C, 30 min in SDS sample buffer; lane 2, 65 °C, 7 min; lane 3, 65 °C, 30 min; lane 4, 100 °C, 7 min (standard conditions); lane 5, 100 °C, 30 min; lane 6, 100 °C, 7 min in sample buffer without 2-mercaptoethanol. Arrows indicate the 100000, 77000 and 71000 *M*ₐ, IROMPS.

PAGE (Fig. 4). A protein similar in mobility to the previously identified 77000 *M*ₐ, IROMP was induced during iron-restriction in all 12 serotypes, although this was difficult to assess in the serotype 10 strain. Larger OMPs from this strain routinely migrated as wavy, diffuse bands making accurate size determinations difficult.

Although the 77000 *M*ₐ, IROMP appeared to be present in all 12 *P. haemolytica* serotypes, several serotype-specific differences were noted in the 100000 and 71000 *M*ₐ, IROMPs. A protein equivalent to the 100000 *M*ₐ, IROMP of the serotype 1 strain was induced in all but the serotype 3 and 9 strains. A 71000 *M*ₐ, protein was present in greater amounts in iron-restricted cells of all serotypes although this difference was modest in the serotype 8 strain. Furthermore, several serotypes expressed additional OMPs in response to iron restriction which were not seen in the serotype 1 strain. A protein of about 60000 *M*ₐ, was present in increased amounts in all but the serotype 1, 10 and 11 strains, and a 95000 *M*ₐ, protein was induced in the serotype 3 to 7 strains. Finally, a novel 43000 *M*ₐ, protein was observed in the serotype 10 strain under iron restriction.

**Immune response to *P. haemolytica* IROMPs**

Western blot analysis was performed in order to determine whether antibodies directed against the three *P. haemolytica* IROMPs could be detected in calves infected with *P. haemolytica*. OMPs from iron-starved cells representing serotypes 1 to 12 were transferred to nitrocellulose paper and reacted with the convalescent serum of a calf which had previously been experimentally infected with the *P. haemolytica* serotype 1 strain B122 (Fig. 5). Antibodies directed against the 100000, 77000 and 71000 *M*ₐ, IROMPs of the homologous serotype 1 strain (Fig. 5, lane 1) could be detected in this serum, as could antibodies against most of the other OMPs. In particular, a constitutive protein of about 50000 *M*ₐ, reacted strongly with this serum. There appeared to be considerable antigenic cross-reactivity between OMPs of the different serotypes of *P. haemolytica* (Fig. 5, lanes 2–12). The 77000 *M*ₐ, IROMP was cross-reactive...
Fig. 4. IROMPs of *P. haemolytica* serotypes 1–12. Strains of serotypes 1–12 were grown in TSB or TSB-D as described in the text and OMP-enriched preparations were separated on SDS-polyacrylamide (8%) gels. Only the relevant portion of the gel is shown. Serotype numbers are given above the lanes: OMPs from iron-restricted cells are shown in the first lane (a) of each pair, while OMPs from iron-replete cells are in the second lane (b). Arrows indicate the 100000, 77000, and 71000 *M*ₐ IROMPs in the serotype 1 strain. Arrowheads indicate the 77000 *M*ₐ IOMP which is conserved between the 12 serotypes. Positions of the *M*ₐ markers are indicated on the right.
Fig. 5. Immunoblot of OMP-enriched preparations from *P. haemolytica* serotypes 1–12. All OMPs were derived from iron-starved cells and reacted with antiserum from a calf experimentally infected with a serotype 1 (strain B122) *P. haemolytica*. Positions of the three serotype 1 (strain B122) IROMPs in lane 1 are indicated on the right. Arrowheads indicate the position of the 77000 *M*ₐ, IROMP in each serotype.

between all serotypes except the serotype 10 strain, while a protein equivalent to the 100000 *M*ₐ, IROMP was recognized by the serotype 1 antiserum in those strains in which it was induced. In addition, the 71000 *M*ₐ, IROMP also demonstrated a pattern of cross-reactivity among all 12 serotypes. Finally, when Western blots of the serotype 1 to 12 strains were probed with antisera derived from calves recovering from natural *P. haemolytica* infections, an identical pattern of reactivity to that shown in Fig. 5 was obtained (results not shown).

**DISCUSSION**

Pathogenic bacteria which invade and colonize an animal host must contend with an almost complete unavailability of iron due to the sequestering of iron as tight complexes with transferrin, lactoferrin, ferritin, haemoglobin and other compounds (Aisen & Listowski, 1980). The ability of pathogens to compete successfully with the host's iron-sequestering mechanism is recognized as being an important, if not critical, aspect of their pathogenicity. *P. haemolytica*, which is readily able to colonize the bovine lung, might therefore also be expected to possess an efficient mechanism of acquiring iron from host stores. A common element of all bacterial iron-acquisition systems examined has been the presence of specific OMPs which are induced by iron starvation and which function as receptors for iron-carrying complexes and in the transport of these complexes through the cell envelope (Braun, 1985; Neilands, 1982). We have shown here that *P. haemolytica* can also respond to an iron-restricted environment by altering the protein composition of its outer membrane. While changes were noted in three OMPs, only one of these, a 77000 *M*ₐ, protein, was apparently completely absent from iron-replete cells. The remaining two proteins, of 100000 and 71000 *M*ₐ, were detectable in iron-replete cells but were synthesized in greatly increased amounts in iron-restricted cells. These specific OMP changes were not due to the coincidental decrease in the growth rate of iron-starved cells, nor were the changes specific for the type of iron chelator used. Chart *et al.* (1986) noted that the choice of chelator used to induce iron-restriction can influence the number of IROMPs observed, but we found an identical IROMP profile in cells grown in 2,2'-dipyridyl and EDDA-treated media.

Induction of the three IROMPs was rapid: they could be detected in *P. haemolytica* outer membranes within 15 min of nutrient shift and at least 15 min prior to a decline in cellular growth rate due to iron deprivation. Synthesis of all three IROMPs appeared to be maximal after 30 min. In contrast, Klebba *et al.* (1982) found that the first of six *E. coli* IROMPs was induced within 24–30 min of shifting iron-replete cells to iron-poor media, and that synthesis of all IROMPs was complete by 90 min. Rapid induction of IROMP synthesis would be necessary to render the bacterial iron-acquisition system fully operable before the existing intracellular
iron stores were depleted. While we have not as yet ascribed a function to any of the \( P. \) \textit{haemolytica} IROMPs, their appearance in cells growing in iron-poor environments suggests that they play some role in mediating the uptake of sequestered iron. The mechanism by which this could occur is not yet clear and further work is necessary to determine whether siderophore-type molecules are involved or whether \( P. \) \textit{haemolytica} IROMPs can remove iron directly from host iron-carrying glycoproteins as is seen with the pathogenic neisseriae (West & Sparling, 1985; Mickelson et al., 1982; Brener & Devoe, 1982). Since \( P. \) \textit{haemolytica} is found primarily on mucosal surfaces, where lactoferrin is a major iron-sequestering glycoprotein, it is possible that sufficient iron for bacterial growth can be acquired from this source.

To assess the \textit{in vivo} expression of the \( P. \) \textit{haemolytica} IROMPs, immunoblotting with convalescent sera from calves both naturally and experimentally infected with \( P. \) \textit{haemolytica} was performed. Antibodies directed against the three \( P. \) \textit{haemolytica} IROMPs, as well as to most of the other high-\( M \), OMPs, were detected in this manner. Similar results have been obtained with a number of Gram-negative pathogens including \( E. \) \textit{coli} (Griffiths et al., 1985), \textit{Neisseria} (Fohn et al., 1987; Black et al., 1986) and \textit{Pseudomonas aeruginosa} (Brown et al., 1984), all of which express immunogenic IROMPs that are reactive with convalescent sera. Our data suggest that the \( P. \) \textit{haemolytica} IROMPs are synthesized \textit{in vivo} by the organism and may be exposed on the surface of the outer membrane, as would be necessary if they were involved in acquiring extracellular iron. In this respect, the IROMPs of \( P. \) \textit{haemolytica} might offer effective targets for vaccine development by inducing antibodies which could prevent the uptake of exogenous iron.

Finally, we have examined the variability of IROMP expression among 12 serotypes of \( P. \) \textit{haemolytica}. Both the 77000 \( M \), and 71000 \( M \), IROMPs were induced by iron-restriction in serotypes 1 to 12, although in the serotype 10 strain this observation is still tentative because of the poor resolution of the higher-\( M \), proteins. Similar anomalous banding patterns of OMPs have been observed in \( E. \) \textit{coli} and are thought to arise from co-migration with lipopolysaccharide (Griffiths et al., 1985). Both these IROMPs also showed antigenic cross-reactivity between serotypes when reacted with whole-cell antisera against the serotype 1 strain of \( P. \) \textit{haemolytica}. Only the 77000–80000 \( M \), IROMP of the serotype 10 strain was apparently not cross-reactive with the 77000 \( M \), serotype 1 IROMP, but it is unclear whether this resulted from co-migration of lipopolysaccharide or indicates some antigenic divergence. The 100000 \( M \), IROMP was expressed in some but not all serotypes. Some serotypes expressed other IROMPs in addition to those described above. It is conceivable that multiple iron-acquisition systems, specific for particular iron sources, are present in \( P. \) \textit{haemolytica}, as has been suggested for \textit{Neisseria gonorrhoeae} (West & Sparling, 1985). The serotype 2 to 12 strains of \( P. \) \textit{haemolytica} are poorly characterized and it remains unclear why only strains of serotype 1 and occasionally serotype 2 are associated with bovine pneumonic pasteurellosis. Our results indicate that any serotype-specific differences in the pathogenicity of \( P. \) \textit{haemolytica} are unrelated to the expression of a particular IROMP. Further studies on IROMPs, including the isolation and characterization of mutants in the IROMP structural genes, should contribute to a better understanding of the pathobiology of this organism.

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P. haemolytica iron-repressible proteins


