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Rapid Identification and Cloning of Bacterial Transferrin and Lactoferrin Receptor Protein Genes

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The sequences of genes encoding the transferrin and lactoferrin receptor proteins from several bacterial species were analyzed for areas of identity in the predicted protein sequences. Degenerate oligonucleotide primers were designed and tested for their ability to amplify portions of the receptor genes. Primer pairs capable of amplifying products of the *tbpA*/*lbpA* or *tbpB*/*lbpB* genes from all species possessing these receptors were identified.

Members of the families *Neisseriaceae* and *Pasteurellaceae* have been shown to possess transferrin (Tf) and lactoferrin (Lf) receptors that are involved in acquisition of iron from these host glycoproteins (7). These receptors are composed of two proteins, Tf/Lf binding protein A (TbpA/LbpA) and Tf/Lf binding protein B (TbpB/LbpB). The presumed importance of these proteins for survival in the host (7) and their utility as vaccine antigens (3, 8, 11) lead to questions regarding (i) the prevalence of the receptor proteins within pathogenic bacterial species and related commensal bacteria and (ii) the heterogeneity of the receptor proteins. It is evident that a rapid, simple, and reliable method of detecting and analyzing receptors in different species and strains would be advantageous for implementing these types of studies. The availability of simple solid-phase binding assays and affinity isolation protocols have undoubtedly been useful, but these approaches have a variety of inherent limitations, including optimizing conditions for receptor expression (12). This prompted the studies outlined below, which involve development of a PCR-based method for amplification of segments of receptor protein genes.

The Tf and Lf receptor genes that have been cloned and sequenced from several different bacterial species (1, 2, 4–6, 10) were used to obtain the predicted sequences of the respective receptor proteins. These sequences were compared to one another by using a variety of alignment programs, with particular emphasis on detecting regions of identity. This analysis identified several regions of identity (Fig. 1 and Table 1) that were potentially useful for the design of degenerate oligonucleotide primers for amplification of DNA between the corresponding sites on the *tbp* and *lbp* genes (Fig. 1 and Table 2). We designed truly degenerate oligonucleotide primers rather than utilizing preferred codon usage tables for the relevant species since we were hoping that the primers may have broad application. Thus, we utilized inosine, which is capable of base pairing with all the potential bases, when any of the four bases

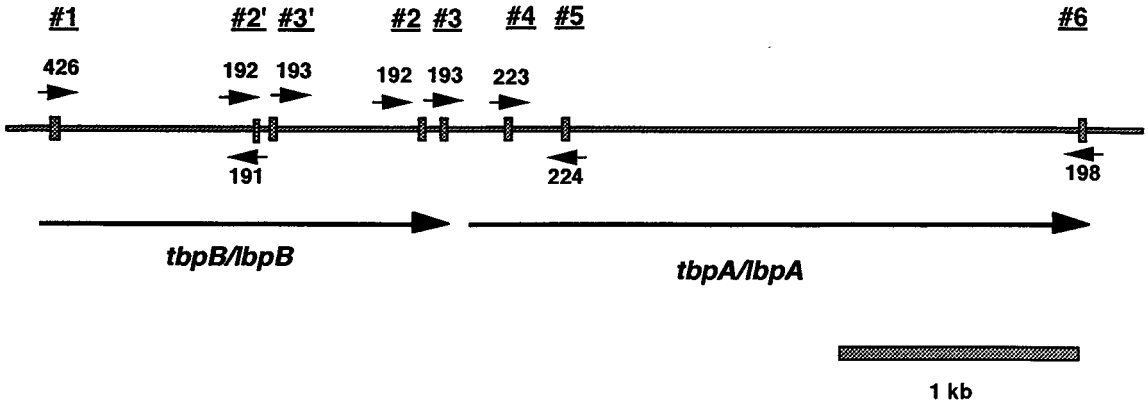

FIG. 1. Location of the regions of homology within the *tbp*/*lbp* operon. The *tbp* or *lbp* genes are represented by the indicated long arrows showing the direction of transcription. The individual regions of homology are indicated by numbers corresponding to those listed in Table 1 and represented by vertical bars on the line representing the DNA. The sites and direction of transcription of oligonucleotide primers listed in Table 2 are indicated by the numbered short arrows. The various segments are drawn to the scale indicated by the hatched bar below.

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TABLE 1. Alignment of conserved predicted protein sequences

Species	TbpB (LbpB)[a]					TbpA (LbpA)		
	Region 1	Region 2′	Region 2	Region 3′	Region 3	Region 4	Region 5	Region 6
Consensus sequence	ACSGGGGSFD	LEGGFYG	VNGGFYG	VVFGAK	VVFGAK	NEVTGLGK	GAINEIEY	AAPGRNY
Neisseria meninigitidis	**L*******	*X***X*	*X*****	X*XX**	******	********	********	*******
Neisseria gonorrhoeae	**L*******	*S***F*	*Q*****	**GS**	******	********	********	*******
Haemophilus influenzae	******-***	*******	***A***	G**S**	******	********	********	**S*****
Actinobacillus pleuropneumoniae	*****K****	*******	VX*****	A**X**	A*****	********	********	*******
Pasteurella haemolytica	***SN*****	*******	FE*****	***AGT	IT**G*	********	********	G*****F
Haemophilus somnus	***S*K****	*****F*	*D*A***	******	G*****	********	********	**R***F
Neisseria meningitidis[b]	na	na	VX*****	na	******	K*******	********	******F

[a] X, a different amino acid in this sequence in proteins from different strains; na, not available.
[b] Amino acid sequences of LbpB and LbpA.

could be present (9). When only two or three bases were possible, we chose to include all possible bases in the synthesis.

The considerable homology among the TbpA and LbpA proteins provided a number of different regions of sufficient identity to be potentially useful for the design of degenerate oligonucleotide primers. Several of the regions were selected on the basis of the high degree of sequence identity, the relative location, and the codon usage for the amino acids (regions 4 to 6, Table 1 and Fig. 1). A set of primers based on the sequences in regions 4 and 5, primers 223 and 224, were particularly useful since they were capable of readily amplifying a 270- to 300-bp fragment (Fig. 2A) of DNA from all species known to possess Tf/Lf receptors (Table 3) as well as from several species in which receptors had not previously been described (*Actinobacillus actinomycetemcomitans*, *Moraxella lacunata*, and *Pasteurella trehalosi*). The combination of primers 223 and 198 (regions 4 and 6, Fig. 1) provided the opportunity to amplify most of the *tbpA*/*lbpA* gene, and an expected 2.5-kb product was produced (Fig. 2B) with DNA from virtually all of the expected species (Table 3). In contrast to the 223-224 primer pair, this combination had a considerable propensity for generation of additional bands and thus required optimization of the PCR conditions. The identity of the 2.5-kb band could readily be verified by its use as a template for amplification with the 223-224 primer combination.

In spite of the low overall homology among TbpB proteins, several regions of localized homology were identified for design of primers (regions 1 to 3′, Fig. 1 and Table 1). An additional region of homology (consensus = CCSNLDY) did not provide useful primers. Two of these regions (regions 2 and 3) were present near the carboxyl terminus of the TbpB or putative LbpB proteins, but similar regions of homology were also observed near the middle of the TbpB proteins (regions 2′ and 3′, Table 1 and Fig. 1). The low degree of identity, the relative position, and the duplication of sites made design of effective primer pairs for amplification of segments of the *tbpB*/*lbpB* genes difficult. The only combination that provided reasonably consistent results with DNA from different species was 426-191 (regions 1 and 2/2′, Fig. 1). The most prominent product usually was approximately 900 bp (Fig. 2C), representing amplification between regions 1 and 2′ (Fig. 1). Although a fragment of the anticipated size was obtained from most of the species tested (Table 3), additional bands in this size range were occasionally also seen, making definitive identification difficult. Unfortunately, the lack of suitable priming sites precluded the use of nested PCR to verify the identity of the PCR products.

As an alternate strategy for amplification of a portion of the *tbpB*/*lbpB* gene, we designed forward primers for regions in the *tbpB*/*lbpB* gene that could be used in combination with reverse primers from the *tbpA*/*lbpA* regions, under the assumption that the genomic organization (*tbpB* preceding *tbpA*) observed to date (7) would be present. Thus, primer 192 (region 2/2′) or primer 193 (region 3/3′) was used in combination with primer 224 (region 5) to amplify a 500- to 700-bp product (Fig. 2D) representing the 3′ end of the *tbpB*/*lbpB* gene, the intergenic region, and the 5′ end of the *tbpA*/*lbpA* gene. This product was amplified from most of the tested species (Table 3), and although the common presence of other products could complicate interpretation, the primer pair 223-224 could verify the identity of the product when it was used as a template in subsequent PCR amplification reactions. It is possible that the failure to amplify this product from several of the species (*Moraxella catarrhalis* and *Moraxella bovis*) might be due to an alternate operonic arrangement of the genes in these species.

The nature of the degenerate primers resulted in amplifica-

TABLE 2. List of oligonucleotide primers

Oligonucleotide no.	Protein		Primer	
	Region	Amino acid sequence[a]	Direction	Nucleotide sequence[b]
426	1	GGSFD	Forward	TTAGGATCCGGIGGI(TA)(CG)ITT(TC)GA
191	2	GGFYG	Reverse	GCGCGGATCCICC(A/G)TA(A/G)AAICCICC
192	2	VXGGFYG	Forward	GTI(T/A)(A/G/C)IGGIGGITT(C/T)TA(T/C)GG
193	3	VVFGAK	Forward	CGCGGATTCGTIGTITT(T/C)GGIGCIAA
223	5	NEVTGXGK	Forward	GCGCAA(T/C)GA(A/G)GTIACIGGIITIGGIAA
224	7	GAINEIEY	Reverse	GCGCTA(TC)TCIAT(TC)TC(AG)TTIATIGCICC
198	8	AAXGRNY/F	Reverse	GCIGCII(G/C)IGCICGIAA(T/C)T(T/A)(T/C)

[a] Amino acid sequence encoded by the sequence of the primer. X, nucleotide sequence encodes several possible amino acids; /, nucleotide sequence encodes the two indicated amino acids.
[b] Nucleotide sequence of primer where parentheses enclose positions in which more than one nucleotide is present, underlined nucleotides indicate noncomplementary bases that may provide a restriction endonuclease cleavage site, and I represents inosine.

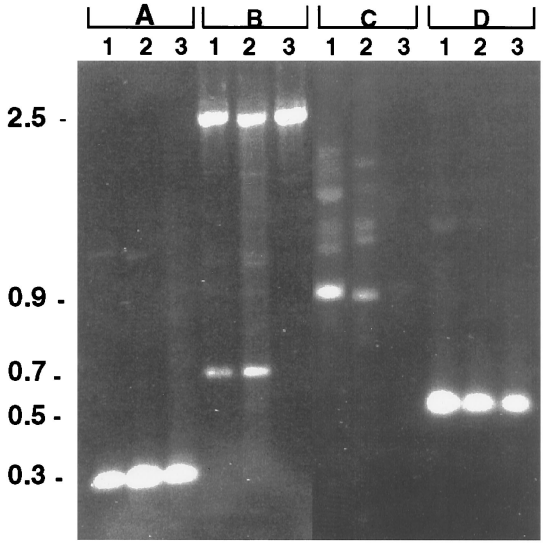

FIG. 2. PCR amplification with *Neisseria meningitidis* genomic DNA and the isolated *tbp* and *lbp* regions as templates. The degenerate primer pairs indicated in lanes A (223-224), B (223-198), C (426-191), and D (193-224) were used in PCR amplification reactions described in the text. The reactions contained genomic DNA from *N. meningitidis* B16B6 (lane 1), the cloned *N. meningitidis* *tbp* region (lane 2) or the cloned *lbp* region (lane 3) as templates. An aliquot from the reaction mixtures was applied to a 1% agarose gel, electrophoresed, and then stained with ethidium bromide. The numbers at the left refer to the sizes of the PCR products in kilobases.

tion of products from both the *tbp* and *lbp* genes (Fig. 2, lanes 2 and 3). Thus, results were relatively straightforward with members of the *Pasteurellaceae*, which only possess Tf receptors (7). In members of the *Neisseriaceae*, which possess both Tf and Lf receptors (7), the primary PCR products usually contained segments from both loci. However, several simple strategies are available to readily isolate segments from the individual loci. Use of the primary PCR product as a probe in Southern blot analysis can identify fragments representing the two loci. The DNA from these regions of the gel can then be used as templates in subsequent PCRs to separately amplify segments from the two loci. Alternatively, we have designed a set of primers internal to 223 that are capable of discriminating between the known *tbpA* and *lbpA* genes, but we are uncertain of their general utility.

Since the degenerate primers used in this study are designed from highly conserved regions from divergent species, they likely represent functionally conserved domains, indicating that this approach should be effective with any species possessing this class of receptor protein. The nature of the receptor proteins indicates that these receptors could be found only in gram-negative species, and to date this mechanism of iron acquisition has been demonstrated only for species from the families *Neisseriaceae* and *Pasteurellaceae*. The simplicity and reliability of this approach should facilitate its rapid application to a wide variety of gram-negative species and will thus give us a true indication of the prevalence of this iron acquisition mechanism. The PCR amplification can be performed on boiled samples from individual colonies, irrespective of the conditions required for growth, likely could even be adapted to work directly on clinical samples, and may have some utility in diagnostic settings.

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TABLE 3. PCR amplification of *tbp*/*lbp* gene fragments from different bacterial species

Bacterial species	PCR product obtained from the indicated regions[a]			
	tbpA/*lbpA*		*tbpB*/*lbpB*	*tbpBA*/*lbpBA*
	223/224 (0.3 kb)	198/223 (2.5–2.8 kb)	426/191 (1.0 kb)	192/224 (0.65 kb)
Neisseria meningitidis	++	+	+/−	+
Neisseria gonorrhoeae	++	+	+/−	+
Neisseria lactamica	++	+	+/−	+
Moraxella catarrhalis	++	+	+/−	−
Moraxella lacunata	++	+	+/−	−
Moraxella bovis	++	+	+/−	−
Pasteurella haemolytica	++	+	+/−	+
Pasteurella trehalosi	++	+	+/−	+
Actinobacillus actinomycetemcomitans	++	+	−	ND
Haemophilus influenzae	++	+	+/−	+
Actinobacillus pleuropneumoniae	++	+	+/−	+
Haemophilus paragallinarum	++	+	−	+/−
Escherichia coli	−	−	−	−
Pseudomonas aeruginosa	−	−	−	−

[a] ++, a predominant product of the anticipated size is readily evident; the identity of this product has been confirmed by sequence analysis in several instances. +, a product of the anticipated size was readily evident, but additional bands were often present; the identity of the 2.5- to 2.8-kb band has been confirmed in many instances by its use as a template for the 223/224 primer pair. +/−, several PCR products in the expected size range were present, and the identities of the products have not been established. −, no product was detected in the anticipated size range.

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